Conversion of Murine Embryonic Stem Cells to Neural Precursor Cells, Astrocytes and Neurons by Differentiation and Corticogenesis and its Characterization

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Contents

List of Tables

Abbreviations

ESC- Embryonic Stem cells

Shh- Sonic Hedge Hog

MEF- Mouse Embryonic fibroblasts

LIF- Leukaemia inhibiting factor

EB's- Embryoid bodies

FBS- Fetal Bovine Serum

DMEM- Dulbecco's Modified Eagle Media

DA- Dopaminergic neurons

NPC's- Neural progenitor cells

PLO- Poly- L- Ornithine

FGF- Fibroblast growth factor

EGF- Epidermal growth factor

TC- Tissue Culture

PFA- Paraformaldehyde

NDS- Normal donkey serum

NGS- Normal goat serum

ECM- Extra cellular matrix

DDM- Defined Default Media

PLL- Poly- L- Lysine

IF- Immunofluorescence

PCA- Principal Component Analysis

List of Figures

Abstract

Conversion of Murine Embryonic Stem Cells to Neural Precursor Cells, Astrocytes and Neurons by Differentiation and Corticogenesis and its Characterization

Hansini Upadhyay

Jennifer E. Phillips- Cremins, PhD and Kara Spiller, PhD

ES cells have been used for many years now to generate specific cell lines of neural cells and they have also emerged as a promising approach in developmental neurobiology, by providing models for neural development.

Several scientists have tried differentiating ES cells into neurons and have often described this process to be cumbersome. However, there are alternative methods available to the differentiation protocol which is shorter in time period than the traditional differentiation methods. This method is called genetic reprogramming. My aim was to compare traditionally differentiated cells to primary cells and characterize them by observing the difference in morphology and their ability to express differentiated cell markers and quantify my results by counting the number of cells expressing the marker using MATLAB.

I chose to focus first on differentiation of murine ES cells to neural progenitor cells (NPC's) as they have the ability to differentiate into many terminally differentiated neural cell types. By optimizing coating and culture conditions, I have established an

enriched population of neural progenitor cells. As mentioned, these cells could then be used to generate a variety of neural cell-types; I chose to optimize differentiation of NPCs down the glial lineage.

ES cells also yield cortical neurons when they undergo a process called Corticogenesis. Corticogenesis is the formation of cortex and this is obtained by using a chemically defined medium in the absence of morphogen but in the presence of a sonic hedgehog inhibitor, Cyclopamine. It causes the cells to directly shift their fate towards neuronal lineage by blocking the Sonic Hedge Hog (Shh) pathway. During this process, neurogenesis is spotted at day 6 which marks the onset of neural lineage formation. Corticogenesis from ESC's recapitulates the most important steps of cortical development, leading to the generation of multipotent cortical progenitors that sequentially produce cortical pyramidal neurons in 3 weeks.

1. Introduction

1.1 Stem cells and its Differentiation

Stem cell research has been a hot subject of research since many years now and scientists have shown interesting and fascinating data related to the potential of stem cell differentiation and its applications in the field of health care. In this project I am differentiating ES cells (v6.5 clone) to neural progenitors, astrocytes and cortical neurons by using growth factors and serum.

ES cells to Neural progenitors is a 4 stage process which first and foremost involves obtaining MEF feeder layers from mice embryos. It is this feeder layers on which the ES cells would be expanded. ES cell expansion is stage 1 of the differentiation protocol. Stage 2 involves formation of 3D aggregates of ES cells called Embryoid bodies. These Embryoid bodies are then cultured 5 to 7 days in ITSFn media (stage 3) which selects for NPC's. Finally NPC's are selected and cultured for 4 days on PLO and Fibronectin coated plates in the presence of EGF and FGF-2 (stage 4). The NPC's are characterised using NPC markers, Sox-2 and Nestin.

To obtain glial lineage, stage 4 NPC's are further cultured for 15 days on PLO coated plates in media containing 5-10% serum. Serum drives the fate of progenitors towards glial lineage, Astrocytes in specific. At the end of 15 days, the cells were characterised using GFAP, an Astrocyte marker.

ES cells also give rise to cortical neurons by a process called Corticogenesis in 21 days. In this step ES cells are cultured as a monolayer in presence of a neural induction media and a sonic hedgehog inhibitor, Cyclopamine. This resulted in the cells to change their fate and proceed to neural lineage. Cyclopamine caused the

cells to form progenitors in 12 days and these were selected by culturing them on PLL and laminin coated dishes. The progenitors yielded cortical neurons in N2/ B27 media after culturing them for 9 days on PLL and laminin substratum. The cells were characterised using a wide range of markers ranging from forebrain and midbrain markers, cortical markers, glial markers and ventral neural markers.

Applications of Stem cells in health care industry are flourishing and there is evidence of its ability to treat neurodegenerative diseases. This has resulted in common man storing baby stem cells (umbilical cord blood and tissues) when the baby is just born. These cells would act as handy source of treatment if the baby develops neurological diseases, diabetes, myocardial infarction, etc. (Lo & Parham, 2009). There are over 200 clinical trials evaluating new born stem cells for treatment of various conditions (CBR).

1.2 Overall objective

The overall objective of this project was to obtain neural progenitor cells that would be differentiated to terminally differentiated Glial cells, Astrocytes and cortical neurons. Terminally differentiated cells were obtained by formation of intermediate structures called Embryoid bodies or by a monolayer culture differentiation method.

The specific aims of this project are as follows:

- Differentiating ES cells to give rise to 3D aggregates at the end of day 4 called Embryoid Bodies which eventually gave rise to neural progenitor cells at the end of 15 days.
- To test these progenitor cells by using a wide range of antibodies specific to NPC's, Astrocytes and Neurons. Characterisation of the differentiated cell type to primary

neural progenitor cells. To also determine the percentage of positive cells by processing images using MATLAB.

- Differentiating NPC's to Astrocytes by culturing the cells in media containing serum for 15 days.
- To test the differentiated astrocytes with GFAP marker specific to astrocytes. Characterisation of the differentiated cell type to primary mouse cortical astrocytes. To also determine the percentage of positive cells by processing images using MATLAB.
- Obtaining Cortical neurons from ES cells by a process called Corticogenesis by treating ES cells with a sonic hedgehog inhibitor, cyclopamine.
- To test the cortical progenitors and neurons at different time periods to analyse differentiation by staining them with a wide range of antibodies specific to NPC's, Astrocytes and Neurons. To also determine the percentage of positive cells by processing images using MATLAB.

2. Background Information

Stem cells are capable of adopting any cell fate during development and they also serve as internal repair system till the person or animal is alive. Stem cells have the capability to give rise to entire organs and researches are trying to generate organs in-vitro. These cells would be used as an application in health care industry to treat a patient with terminal diseases. Stem cells can be classified based on different parameters based on cell of origin, potency, etc.

Classification of stem cells based on cell of origin:

i) Adult/ Somatic stem cells- They are the cells derived from non- germ cells, egg and sperm. These cells are responsible for giving rise to multipotent cells. Previous studies have shown the development of somatic fates like hematopoietic, neuroectodermal and mesodermal (Bain, Kitchens, Yao, Huettner, & Gottlieb, 1995) (Wober & Boheler, 1999).

ii) Embryonic stem cells- They are the cells derived from the inner cell mass of developing blastocyst and can proliferate extensively in- vitro.

Classification of Stem cells based on potency:

i) Pluripotent: These cells are capable of differentiating into all the cell types of the body excluding the placenta.

ii) Totipotent: These cells are capable of differentiating into all the cell types of the body including the placenta.

iii) Multipotent: These cells are capable of differentiating into specific cell types.

All Stem cells are totipotent for the first few divisions after which they become pluripotent.

Differentiation Potentiality of ESC's (Meregalli, Farini, & Torrente, 2011)

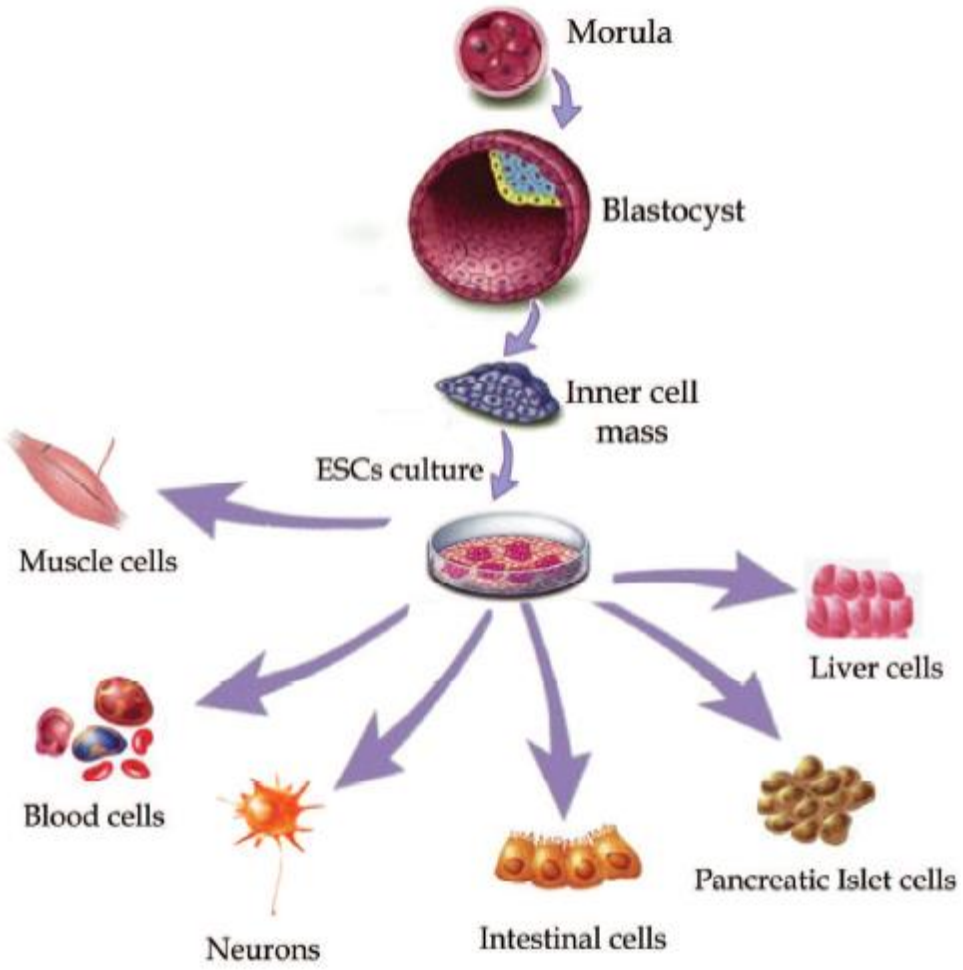


Figure 1: ESC's Differentiation (Meregalli, Farini, & Torrente, 2011)

In order to understand the developmental transition of stems cell, it is necessary to control ES cell differentiation before they differentiate into specific cell types. ES cell differentiation is achieved by two methods:

i) Formation of Embryoid bodies (EB's)

In this method, 3D clusters of pluripotent ES cells are formed which have the capability to differentiate into cells derived from the three germ layers- Ectoderm, Endoderm and Mesoderm.

ii) Monolayer culture method

In this method, ES cells are coated at a specific density and are then exposed to neural Induction media thereby facilitating their conversion to Neuronal lineage.

Expansion of ES cells is done on Mouse Embryonic fibroblasts (MEF) feeder layers. MEF's are obtained from day 13 or 14 pregnant mice embryos and cultured on gelatin. These cells cannot be expanded indefinitely as they exit the cell cycle after passage 5. MEF feeder layers are inactivated using Mitomycin C, an antitumor antibody that inhibits the proliferation of feeder cells by inhibiting DNA synthesis and nuclear division. It does this by cross-linking complementary strands of DNA which prevents separation of complementary DNA strands and inhibits DNA replication. Cells are arrested at the S and G2/M phases of the cell cycle (Kang, Chung, Yoo, Choi, & Yu, 2001).

ES cells are then cultured and expanded on Mitomycin C inactivated MEF's. Care is taken that the colonies are passaged before they touch each other. EC's lost their pluripotency and started to differentiate if the colonies were touching each other.

ES cell differentiation involving formation of Embryoid Bodies includes several stages: Expansion of undifferentiated ES cells (stage1), generation of EB's (stage 2),

selection of NPC's (stage 3), expansion of NPC's (stage 4) and differentiation (stage 5) (Figure 2) (Lee, Lumelsky, Studer, Auerbach, & McKay, 2000).

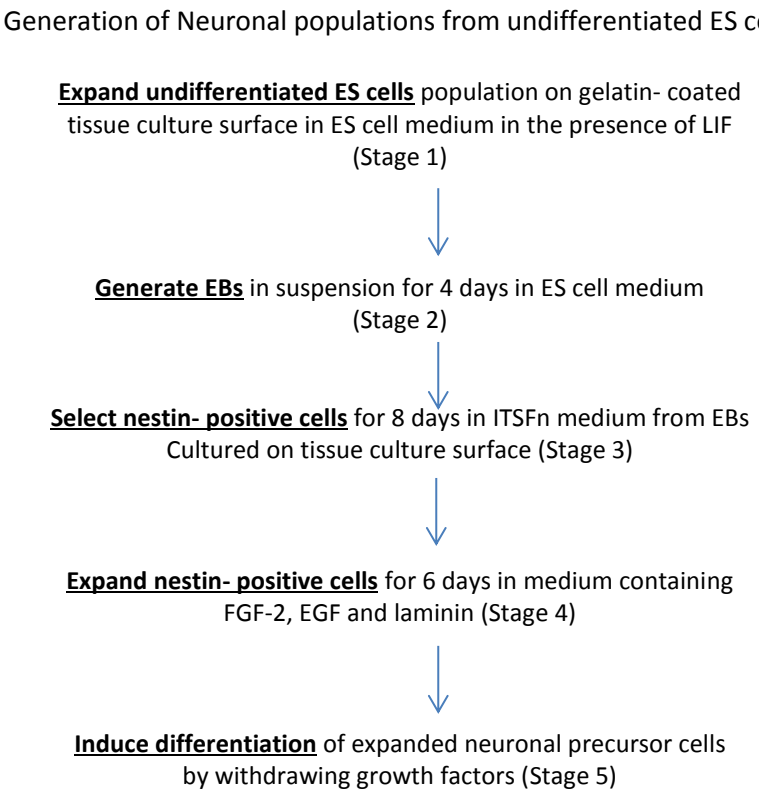


Figure 2: General scheme of ES cell Culture (reproduced from Lee, Lumelsky, Studer, Auerbach, & McKay, 2000)

Neural progenitor cells (stage 4 cells in the differentiation scheme) are cells that can differentiate into specific neural cell types. As the name suggests, they are precursor cells to the target cell type. Neural progenitor cells differ from stem cells for a simple reason that progenitors replicate for a specific number of divisions as compared to ES cells that replicate indefinitely.

Expansion of NPC's is brought about by growth factors, FGF- 2 (Wernig, et al., 2008) and EGF (Meissner, et al., 2008). FGFs constitute a large family of proteins involved in many aspects of development including cell proliferation, growth and

differentiation. They act on several cell types to regulate diverse physiologic functions including angiogenesis, embryonic development, metabolic regulation, cell migration, pattern formation, neurotrophic effects, cell growth, and tissue repair. FGF family activities are mediated by receptor tyrosine kinases and have been implicated in progression (R & D Systems, 2013). Significant numbers of in-vitro and in-vivo biological effects have been ascribed to EGF and other members of the EGF family. EGF is better known for its ability to promote proliferation and differentiation of mesenchymal and epithelial cells. In vitro, EGF is a mitogen for fibroblasts, epithelial and endothelial cells, and promotes colony formation of epidermal cells in culture (R & D systems, 2013).

NPC's require a substratum and ECM protein for the cells to attach to the TC plates in vitro. Different substratum and ECM protein conditions are currently used to expand and culture stage 4 NPC's. (Wernig, et al., 2008) used PLO and fibronectin whereas (Lujan, Chanda, Henrik, Sudhof, & Wernig, 2011) used PLO and laminin as a substratum. Also there has been evidence on using higher concentration of substratum if culturing the cells on a glass coverslip vs polystyrene TC surface (EMD Millipore, 2012). Hence a combination of 4 different substratum + ECM protein conditions was tried to evaluate the best parameter for stage 4 NPC expansion and culture.

We all are aware that neural progenitor cells are capable of differentiating into specific cell types. These cell types can be Neurons, Astrocytes, Oligodendrocytes, etc. Out of all the cells types, I am interested in astrocytes from the glial lineage and cortical neurons from neural lineage.

Conversion of NPC's to Astrocytes was brought about by withdrawing the growth factors and exposing the cells to media containing serum (Lujan, Chanda, Henrik, Sudhof, & Wernig, 2011) for 6 to 8 days. It can also be obtained by exposing stage 4 NPC's to 5% serum conditions for 5 days (Meissner, et al., 2008). However, various researches have used different time period for obtaining astrocytes. Also, there is no evidence available if laminin used for culturing NPC's is used as a growth factor or as an ECM protein. Hence I propose various conditions using different serum concentrations and coating conditions with and without laminin to differentiate and to maintain astrocytes. The cells were exposed to serum for 15 days as there is evidence that morphology shift is spotted first during differentiation however the protein transformation takes a little longer. 15 days was an extreme limit and a concrete time period to obtain astrocytes based on the available evidence.

Significant research has been done to treat neurodegenerative diseases and pioneers have been able to obtain dopaminergic neurons (Wernig, et al., 2008) and serotonergic neurons (Lee, Lumelsky, Studer, Auerbach, & McKay, 2000). These studies have contributed towards understanding Parkinson's disease and many more Neurological disorders. There are many more types of neurons and not all these types of neurons have been fully explored. I was interested in focussing on cortical neurons from ES cells as there has been very little research done on cortical neurons.

The cerebral cortex is composed of a wide variety of Neuronal subtypes constituting 80% of pyramidal neurons generated from cortical progenitors. Each neuronal subtype is characterised by morphology, its position, marker expression and pattern of connectivity. However the understanding of mechanisms involved has been

difficult to study due to the on-going development of the complex structure, cortex. One way to understand corticogenesis is by ES cells (Gaspard, Gaillard, & Vanderhaeghen, 2009). ES cells have emerged as a powerful tool for developmental biology, allowing the in vitro recreation of events of organogenesis in controlled and reproducible conditions, as well as the directed differentiation of specific neuronal populations, such as spinal motor neurons (Wichterle, Lieberam, Porter, & Jessell, 2002) or midbrain dopaminergic neurons (Lee, Lumelsky, Studer, Auerbach, & McKay, 2000). Differentiation of ES cells to cortical neurons is obtained by culturing the ES cells as a monolayer in the presence of neural induction media. It is this neural induction media that drives the ES cells to change their fate to cortical neurons.

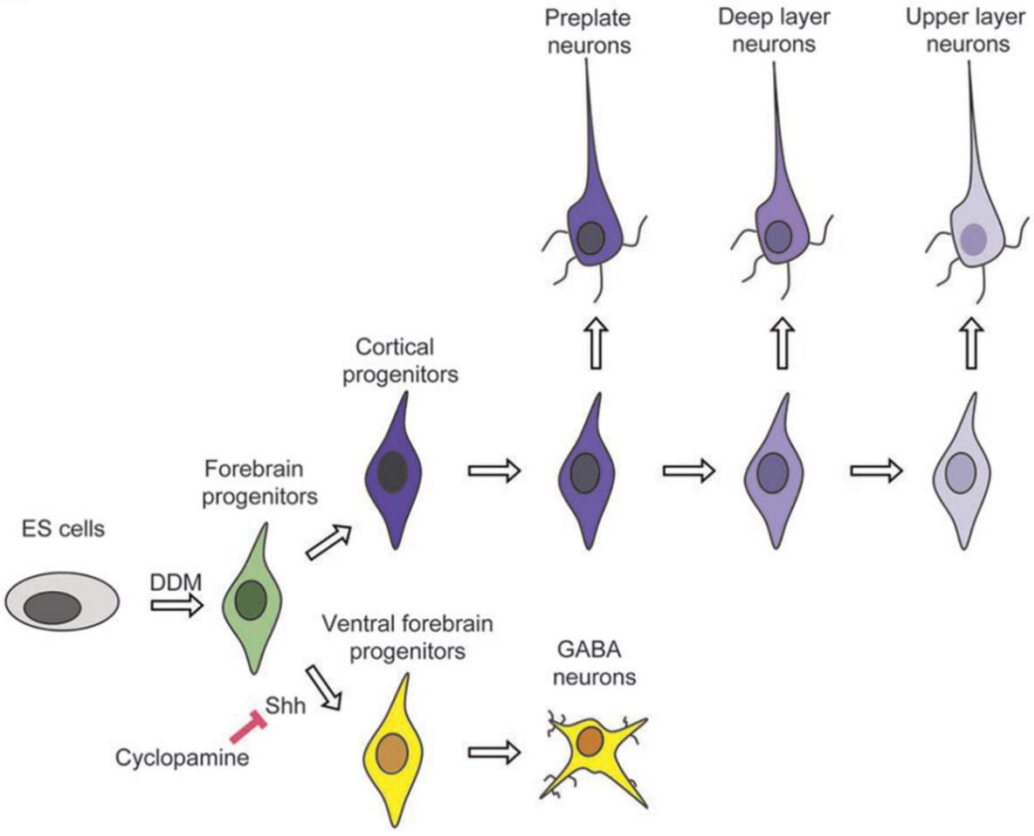


Figure 3: Schematic representation of in- vitro corticogenesis (Gaspard, Gaillard, & Vanderhaeghen, 2009)

Using purely in vitro protocols, ES cells can be very efficiently differentiated into cortical progenitors and subsequently cortical pyramidal neurons, following similar pathways as in vivo (Gaspard, et al., 2008). This was achieved by thawing ESCs and expanding them in ES media. When reaching suitable density, ESCs are passaged and seeded onto gelatin coated dishes. This day is considered as differentiation day 1. ESCs are cultivated in ES media for one day before ES media is changed for DDM (differentiation day 0). Change to DDM marks early differentiation and neural induction. From differentiation days 2 to 10, cyclopamine is added to DDM to induce dorsalization of progenitor cells. Neurogenesis starts at day 6 and will proceed until day 21. At differentiation day 12, cell culture that now contains a majority of progenitors and neurons are seeded onto poly-L-lysine and laminin coated coverslips in N2/B27 media. Cell culture is stopped on 2 and 9 days later, to analyse the identity of progenitors and neurons (options A and B, respectively). (Gaspard, Bouschet, Herpoel, Naeije, Ameele, & Vanderhaeghen, Generation of Cortical Neurons from embryonic stem cells, 2009)

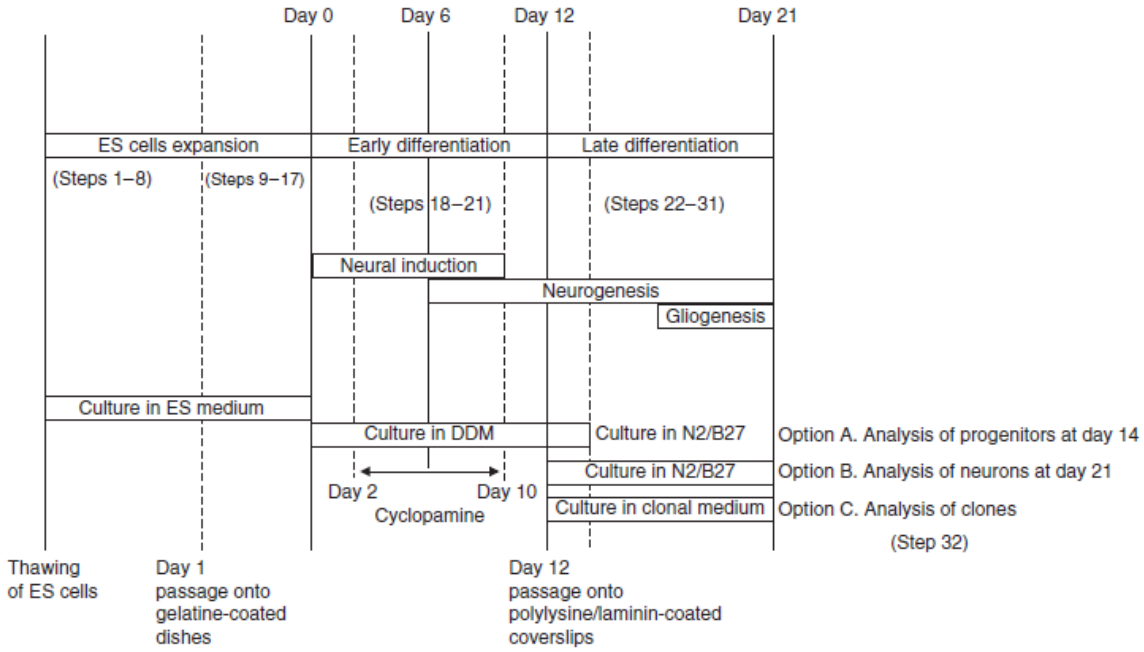


Figure 4: Timetable of Corticogenesis from Embryonic stem cells (Gaspard, Bouschet, Herpoel, Naeije, Ameele, & Vanderhaeghen, Generation of cortical neurons from mouse embryonic stem cells, 2009)

Characterisation of all the cell types, NPC's, Astrocytes and Neurons was done by immunocytochemistry using a wide range of antibodies listed below in the table:

Table 1: List of antibodies used for characterisation of cells at different stages

ANTIBODY	**Function**	**MARKER OF CELL TYPE**
Oct-4	Transcription factor	ES cell
Sox-2	Transcription factor	ES cell, Neural progenitors
Nestin	Intermediate filament protein	Neural progenitors
β- III- Tubulin/ Tuj 1	Structural protein	Neurons
GFAP	Intermediate filament protein	Astrocytes
MAP2	Microtubule associated protein	Mature Neurons
Nkx2.2	Transcription factor	Ventral progenitors
OTX1	Transcription factor	Forebrain and midbrain progenitors
Pax6	Transcription factor	Cortical and dorsal thalamic progenitors
VGAT	Transporter protein	GABA Neurons
VGluT1	Transporter protein	Glutamergic neurons
VGluT2	Transporter protein	Glutamergic neurons

3. Materials and Methods

3.1 Preparation of Feeder layers

Feeder layers were isolated from mice embryos and were used to expand ES cells. Mouse embryos were obtained from the neuron core facility at University of Pennsylvania.

3.1.1 Preparation of Gelatin coated plates

10 ml of 0.1% Gelatin solution (Millipore, ES-006-B) was used to coat 100 mm dishes and incubated for atleast 30 minutes in the tissue culture hood. Gelatin acts as a feeder layer for the MEF's (EMD Millipore) (Novus Biologicals, 2014).

3.1.2 Dissection of Mice

Pregnant mice were sacrificed on Day 13 or 14 post coitum by dissecting the uterine horns using sterile equipments. Dissection step was performed at the neuron core facility of University of Pennsylvania and embryos were collected in PBS (Michalska, 2007) and transported to the Cremin's laboratory. All the further steps for isolation of mouse embryonic fibroblasts were performed at the Cremin's laboratory.

3.1.3 Isolation of embryonic fibroblasts

Uterine horns were rinsed with 70% v/v ethanol and PBS. Using aseptic conditions, each embryo was separated from its placenta and embryonic sac. After obtaining

individual embryos, the head and other non-visceral (red organs) were cut out. The remaining pieces were rinsed in PBS so that they are devoid of blood. All the tissues were minced using a sterile blade to be as fine as possible and able to be pipetted. The minced tissue was incubated with trypsin / EDTA and 100 KU units of DNAse I (Sigma- Aldrich, D4527) for 5 minutes. Further, the contents were incubated in a water bath for 15 minutes followed by dissociation of tissues using a pipette every 5 minutes. The trypsin was inactivated and the contents were centrifuged at 1000 rpm or 300G for 5 minutes. The pellet was resuspended with warm MEF media and seeded on 0.1% gelatin coated dishes (Michalska, 2007). MEF media (Novus Biologicals, 2014) constituted of DMEM- Hi Glucose (Caisson labs, DML10-500), 10% v/v FBS (Atlanta Biologicals, S11550), 1% v/v L- Glutamine (Gibco, 25030-081), 1% v/v Penicillin- Streptomycin (Sigma, P0 781-100), 1% v/v non-essential amino acids (Millipore, TMS-001-C).

3.1.4 MEF passaging and expansion

MEF cells were washed with PBS without ions and then trypsinized with 0.05% trypsin (HyClone, SH30236.02) for 5 minutes at 37°C. Trypsin was inactivated using media containing serum and spun down at 1000 rpm for 5 minutes. Cell pellet was resuspended in MEF media and seeded onto 0.1% gelatin coated plates. MEFs will grow slower with each passage and became senescent after 20 cell divisions (~5 to 7 passages) (Michalska, 2007).

3.1.5 MEF Cryopreservation

Both MEFs and feeder cells can be frozen and stored in liquid nitrogen. When thawed, MEF's remain viable and can be further expanded or used for the preparation of feeder layers (Michalska, 2007). MEF's were trypsinized by using the procedure in cell passaging and resuspended in freezing media containing MEF media, DMSO (10% final volume) and FBS (20% final volume) (Lanctot, 2004).

3.1.6 Thawing MEF cells from liquid nitrogen

A vial containing MEF cells was thawed in 37° C water bath until half of the contents were in liquid state. The contents from the cryovial were mixed with 10ml warm MEF media and spun down at 1000 rpm for 5 minutes. The pellet was resuspended with MEF media and expanded (EMD Millipore) (Novus Biologicals, 2014).

3.1.7 MEF inactivation using Mitomycin C

MEF inactivation can be done by γ irradiation or Mitomycin C (Fisher Scientific, BP 25312). MEF's cells were expanded in MEF media on gelatin coated plates. These MEF's were treated with 10ug/ml of Mitomycin C for 2 hours and trypsinized to be seeded on gelatin coated plates at the density of $2* 10^6$ cells per 100 mm dish (EMD Millipore) (Michalska, 2007). The plates can be stored in the incubator at 37°C for over 3 weeks with regular changing of media.

3.2 ES cell expansion and cryopreservation

ES cells (v6.5 cell line, Novus Biologicals, NBP1-41162) were cultured using DMEM high glucose (Caisson labs, DML 10-500) supplemented with non-essential amino acids (Millipore, TMS-001-C), Penicillin- Streptomycin (Sigma, P0781-100), 100X β-mercaptoethanol (Millipore, ES-007-E), FBS (HyClone, SH30070.03), L- Glutamine (Gibco, 25030-081)and 10^3 units/ml LIF (Millipore, ESG1107) (Novus Biologicals, 2014).

3.2.1 Thawing ES cell from liquid nitrogen

A tube of $2*10^6$ ES cells were thawed in 37° C water bath until half of the contents were in liquid state. The contents from the cryovial were resuspended with 10ml of warm ES cell media and spun down at 1000 rpm for 5 minutes. The pellet was resuspended in ES cell media and seeded at 2-3* 10^6 cells / 100 mm dish (EMD Millipore) containing Mitomycin C inactivated MEF feeder cells. (Novus Biologicals, 2014).

3.2.2 ES Cell expansion

ES cell expansion was done using ES cell media (Novus Biologicals, 2014). The cells were grown on Mitomycin C inactivated MEF's/ feeder layers maintaining the pluripotency of the cells. Colonies were passaged before they were at the risk of touching each other. ES cells would lose their pluripotency if the colonies touch each other when they are being cultured.

3.2.3 Cell Passaging

ES cells were washed with PBS without ions and then trypsinized with 0.05% trypsin HyClone, SH30236.02) for 5 minutes at 37°C. Trypsin was inactivated with media containing serum and spun down at 1000 rpm for 5 minutes. Cell pellet was resuspended in ES cell media and seeded onto the feeder layers (Novus Biologicals, 2014).

3.2.4 Cryopreservation

ES cells were washed with PBS without ions and then trypsinized with 0.05% tryspin (HyClone, SH30236.02) for 5 minutes at 37°C. Trypsin was inactivated with media containing serum and spun down at 1000 rpm for 5 minutes. Cell pellet was resuspended in one part ES cell media and one part freezing media. The freezing media composition was 60% DMEM (Caisson labs), 20% DMSO (Fisher Scientific, EC-200-664-3), 20% FBS (HyClone) (Novus Biologicals, 2014). Cells were stored in cryovials and frozen down for future use in liquid nitrogen.

3.3 Early Differentiation

Before the ES cell colonies were at the risk of touching, they were passaged onto gelatin coated plates. Two to three days later, or before the colonies are at risk of touching, the cells were passaged off gelatin and seeded on 100 mm bacterial grade dishes at the density of $2*10^6$ cells per dish for EB formation. EB's are allowed to form in ES media without LIF on a rotary set at 40 rpm in an incubator for 3- 4 days and media is replenished every 2 days. After 4 days, EB's are diluted to adhere on tissue culture plates overnight in ES media without LIF. Neuronal precursors were

then selected by culturing the cells in DMEM/F-12 media containing insulin – Transferrin- sodium selenite supplement (1X)(Roche, 11074547001), fibronectin (2.5 µg/ml) (Corning, 356008), Penicillin- Streptomycin (Sigma, P0 781-100) (ITSFn media) for 7–10 days (Wernig, et al., 2008).

3.4 Neural progenitors

Cells were subsequently dissociated by trypsin (0.05%), and neuronal precursors were expanded and patterned for 4 days after plating onto fibronectin (1µg/ml) (Corning, 356008) and poly-l-ornithine (15 µg/mL)(Sigma, P4957-50ML) coated plates at a density of 75,000 cells per cm^2 in DMEM/F-12 media containing insulin – Transferrin- sodium selenite supplement (1X)(Roche, 11074547001), progesterone (20 nM) (Sigma, P0130-25G), putrescine (100 nM) (Sigma, P7505-25G), Penicillin-Streptomycin (Sigma, P0 781-100), laminin (1 µg/ml) (Sigma, L2020-1MG), basic fibroblast growth factor (FGF2) (10 ng/ml) (R & D Systems, 234-FSE-025) (Wernig, et al., 2008)and EGF (20ng/ ml) (R & D Systems, 236-EG-200) (Meissner, et al., 2008).
Before the Neural progenitors were exposed to differentiation conditions, the cells were subjected to different coating conditions using the same stage 4 media to determine the best coating condition in terms of cell survival and cell number.

3.4.1 Coating conditions

The effect of reversing the ECM protein as coating reagent vs using it as a growth factor was studied by culturing the NPC's under various conditions. There were 4 conditions that were tried using combinations and they are:

a) Poly- L-Ornithine (15µg/ml) and fibronectin (1µg/ml) for coating and laminin (1µg/ml) added as a growth factor.
b) Poly- L-Ornithine (75µg/ml) and fibronectin (1µg/ml) for coating and laminin (1µg/ml) added as a growth factor.
c) Poly- L-Ornithine (15µg/ml) and laminin (1µg/ml) for coating and fibronectin (1µg/ml) added as a growth factor.
d) Poly- L-Ornithine (15µg/ml) and laminin (1µg/ml) for coating and no additional growth factor.

3.5 Differentiation

Neural precursor cell lines were sequentially passaged and propagated in the presence of EGF and FGF2 and then treated with various growth factors to obtain Astrocytes and Neurons. Seeding density of NPC's has to be atleast 75,000 cells/ cm^2.

3.5.1 Astrocytes

Differentiation to astrocytes was induced by growth factor (EGF and FGF2) withdrawal and addition of 5- 10% FBS (Meissner, et al., 2008) in DMEM high Glucose containing Penicillin- Streptomycin for 15 days. Media was replenished every 2 days. Astrocytes were stained for GFAP protein post fixation. Positive control was primary astrocytes obtained from mouse cortex and negative control was ES cells.

3.5.2 Coating Conditions

Different coating and serum conditions were used to identify the correct growth and culture conditions for astrocytes. There were 6 conditions tried and are listed below:

a) Poly- L-Ornithine (15µg/ml) with 10% serum
b) Poly- L-Ornithine (15µg/ml) with 5% serum
c) Poly- L-Ornithine (15µg/ml) with 5% serum and Laminin (1µg/ml) as a growth factor
d) Poly- L-Lysine (50µg/ml) with 10% serum
e) Poly- L-Lysine (50µg/ml) with 5% serum
f) Poly- L-Lysine (50µg/ml) with 5% serum and Laminin (1µg/ml) as a growth factor

3.6 Differentiation of ES cells to Neurons by Corticogenesis (Gaspard, Bouschet, Herpoel, Naeije, Ameele, & Vanderhaeghen, 2009)

ES cells to cortical neurons were generated on corticogenesis protocol based on Generation of Cortical Neurons from embryonic stem cells paper (Gaspard, Bouschet, Herpoel, Naeije, Ameele, & Vanderhaeghen, 2009).

ES cells were passaged on gelatin after expansion on feeder layers. The cells were trypsinized and titurated thoroughly with pipette tips so as to obtain a single cell suspension.

Cells were adjusted to a concentration of 30,000 cells/ ml with ES media and 5 ml of suspension (i.e., 150,000 cells) were seeded onto a 60-mm gelatin-coated dish. This provided a plating density of approx. 5,000 cells/ cm^2. The cells were cultured for 12 days in an incubator at 37 °C, 5% CO_2.

3.6.1 Early Differentiation

DDM media consisting of DMEM/ F-12+ Glutamax (Life Technologies, 10565018), N_2 supplement (Invitrogen, 17502048), NEAA, Sodium Pyruvate (Sigma, P4562-25G), BSA (Fisher, BP1600-100), BME, Penicillin – Streptomycin was used to culture the cells. This day was considered as differentiation day 0. After 2 days (differentiation day 2) DDM supplemented with cyclopamine (EMD Millipore, 239803-1MG) at the concentration of 1uM was added. The cells were cultured for 48 hours in an incubator at 37 °C, 5% CO_2 until differentiation day 10. On differentiation day 10, the medium was replaced with DDM only (no cyclopamine). Excessive cell death was observed as the day progresses and hence washing with PBS before adding new media is highly recommended (Gaspard, Bouschet, Herpoel, Naeije, Ameele, & Vanderhaeghen, 2009).

3.6.2 ESC-derived neural progenitor's dissociation and late differentiation

On differentiation day 12, 15-mm coverslips were coated in 24-well plates with poly-L-lysine (Sigma, P4707-50ML) and laminin and incubated for 2 hours at room temperature.

Cells were trypisinized and were resuspended with 1 ml of N2/B27 by pipetting with filter tips and Pasteur pipettes respectively. N2/B27 media was used for culturing cells post seeding on coverslips and it consists of Neurobasal media (Neurobasal (Invitrogen, 21103049), B27 supplement minus vitamin A (50X) (Invitrogen, 12587010), Glutamine, Penicillin- Streptomycin) and DDM in the ratio 1:1.

Cells were seeded onto pre-coated coverslips at different densities for progenitor and neuronal analysis at day 14 and 21 respectively using IF staining. However the cells were extremely overcrowded on day 21 and hence were trypsinized on day 21 and reseeded on PLL and laminin coated coverslips at various densities. Analysis of progenitors and neurons was now done on day 21, 23 and 27 respectively.

Option A- Progenitor Analysis at differentiation day 23

250* 10^3 - 500 * 10^3 cells were seeded per well of a 24 well plate containing coated coverslips in N2/B27 medium.

The cells were cultured for 2 additional days in an incubator at 37 °C, 5% CO_2 and then fixed with 4% PFA.

Option B- Neuronal analysis at differentiation day 21 and day 27

125* 10^3 - 250 * 10^3 cells were seeded per well of a 24 well plate containing coated coverslips in N2/B27 medium.

The cells were cultured for 9 additional days in an incubator at 37 °C, 5% CO_2.

N2/B27 medium was changed every 2 days until stopping the experiment for analysis (differentiation day 21) (Gaspard, Bouschet, Herpoel, Naeije, Ameele, & Vanderhaeghen, 2009).

3.7 Immunocytochemistry

3.7.1 Immunocytochemistry for NPC's and Astrocytes

For immunofluorescent staining, cells on coverslips were fixed with 4% PFA for 10 minutes. Fixation was followed by washing them 3X with PBS (ions). Blocking was done using PBS, 10% normal donkey serum (NDS) or normal goat serum (NGS) and 0.1% Triton X-100 for 1 hour. Cells were stained with primary antibodies overnight (Wernig, et al., 2008). Coverslips were then incubated overnight at 4°C on a rocker with primary antibodies diluted in PBS, 10% NDS/NGS, 0.1% Triton X-100. The primary antibodies used were: goat anti- Sox-2 (1:100, Santa Cruz Biotechnology, SC 17320), rabbit anti- Nestin (1:1000, Covance, PRB-315C), rabbit anti-GFAP(1:1000,Sigma Aldrich, SAB4300647), mouse monoclonal anti- MAP2 clone (1:500, Sigma Aldrich, M1406), mouse β III tubulin/ TujI (1:1000, BioLegend MMS-435P), rabbit anti- VGAT (1:3000, Synaptic Systems, 131003), rabbit anti- VGLuT1 (1:2000, Synaptic Systems, 135303) (Gaspard, Bouschet, Herpoel, Naeije, Ameele, & Vanderhaeghen, Generation of cortical neurons from mouse embryonic stem cells, 2009). The coverslips were washed 2X with PBS and subsequently incubated in fluorescent-labelled secondary antibodies (Life Technologies) in PBS and 10% NDS/NGS for 2 hours at room temperature. After rinsing for 2X with PBS, coverslips were mounted onto slides with ProLong gold antifade reagent with DAPI (Life technologies, P36935) (Lujan, Chanda, Henrik, Sudhof, & Wernig, 2011).

3.7.2 Immunocytochemistry for Cortical Neurons

For immunofluorescent staining, cells on coverslips were fixed with 4% PFA for 10 minutes. Fixation was followed by washing them 3X with PBS (ions). Blocking was

done using PBS, 10% normal donkey serum (NDS) or normal goat serum (NGS), 0.1% Triton X-100 for 1 hour. Cells were stained with primary antibodies overnight (Wernig, et al., 2008). Coverslips were then incubated overnight at 4°C on a rocker with primary antibodies diluted in PBS, 10% NDS/NGS, 0.1% Triton X-100. The primary antibodies used were: goat anti- Sox-2 (1:100, Santa Cruz Biotechnology, SC 17320), rabbit anti- Nestin (1:1000, Covance, PRB-315C), rabbit anti- GFAP(1:1000,Sigma Aldrich, SAB4300647), mouse monoclonal anti- MAP2 clone (1:500, Sigma Aldrich, M1406), mouse β III tubulin/ Tuj I (1:1000, BioLegend MMS-435P), rabbit anti- PAX-6 (1: 2500, Covance, PRB- 278P), mouse anti- OTX1 (1: 10, DSHB), rabbit anti- VGAT (1:3000, Synaptic Systems, 131003), rabbit anti- VGLuT1 (1:2000, Synaptic Systems, 135303), rabbit anti- VGLuT2 (1:2000, Synaptic Systems, 135403) (Gaspard, Bouschet, Herpoel, Naeije, Ameele, & Vanderhaeghen, 2009). The coverslips were washed 2X with PBS and subsequently incubated in fluorescent-labelled secondary antibodies (Life Technologies) in PBS and 10% NDS/NGS for 2 hours at room temperature. After rinsing for 2X with PBS, coverslips were mounted onto slides with ProLong gold antifade reagent with DAPI (Life technologies, P36935) (Lujan, Chanda, Henrik, Sudhof, & Wernig, 2011).

4. Results

4.1 MEF's

MEF's obtained from harvest were expanded upto Passage 5. Images on gelatin coated plates were taken at various objectives to check their morphology and density for survival (Figure 5).

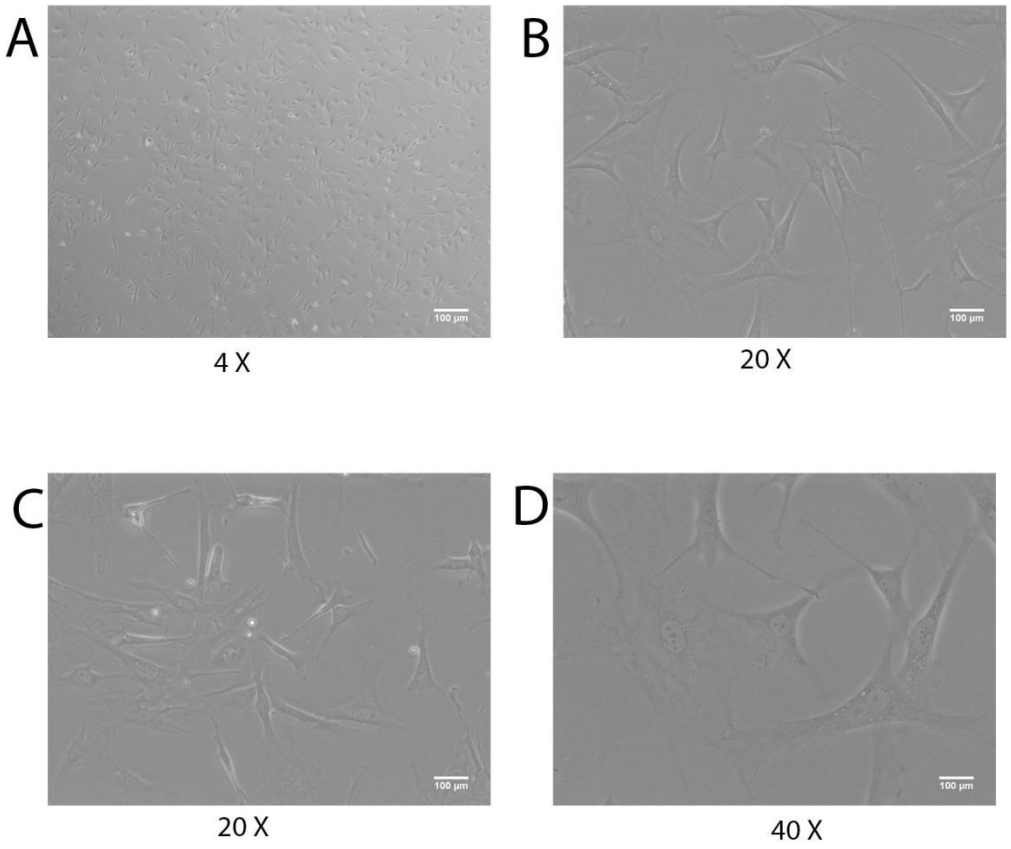


Figure 5: Phase images of MEF's on Gelatin coated plates. (A) MEF image at 4X (B- C) MEF image at 20X (D) MEF image at 40X

4.2 ES cells

Once the MEF's were expanded, ES cell clones, v6.5, were expanded on mitomycin C inactivated MEF surfaces. ES cells always grew as clumps (Figure 6) and formed 3D aggregates by pilling one on top of the other while dividing.

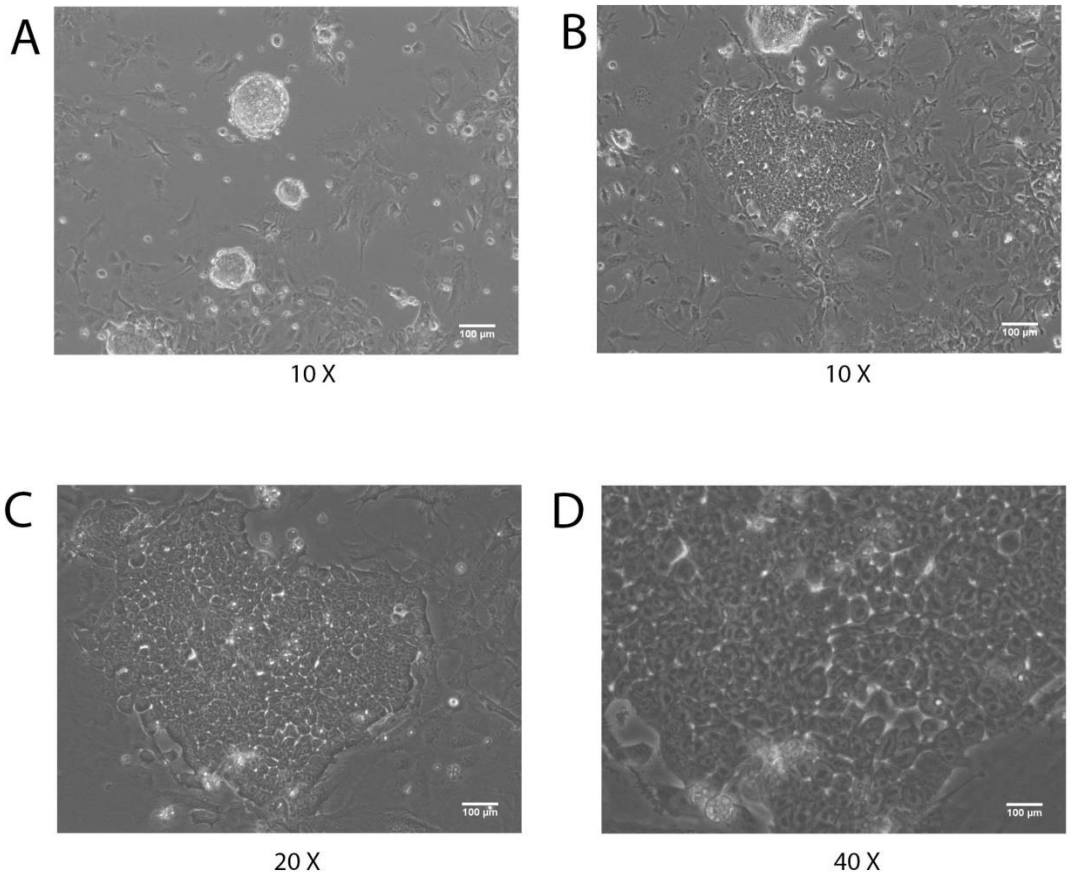


Figure 6: ES cell colonies at (A, B) 10X (C) 20X and (D) 40X

Before the ES cells were subjected to differentiation under the effect of various growth factors, the cells were tested for their pluripotency using Oct- 4 marker. The cells tested positive as seen (Figure 7). It is necessary to check the pluripotency of ES cells before differentiating them into specific cell types.

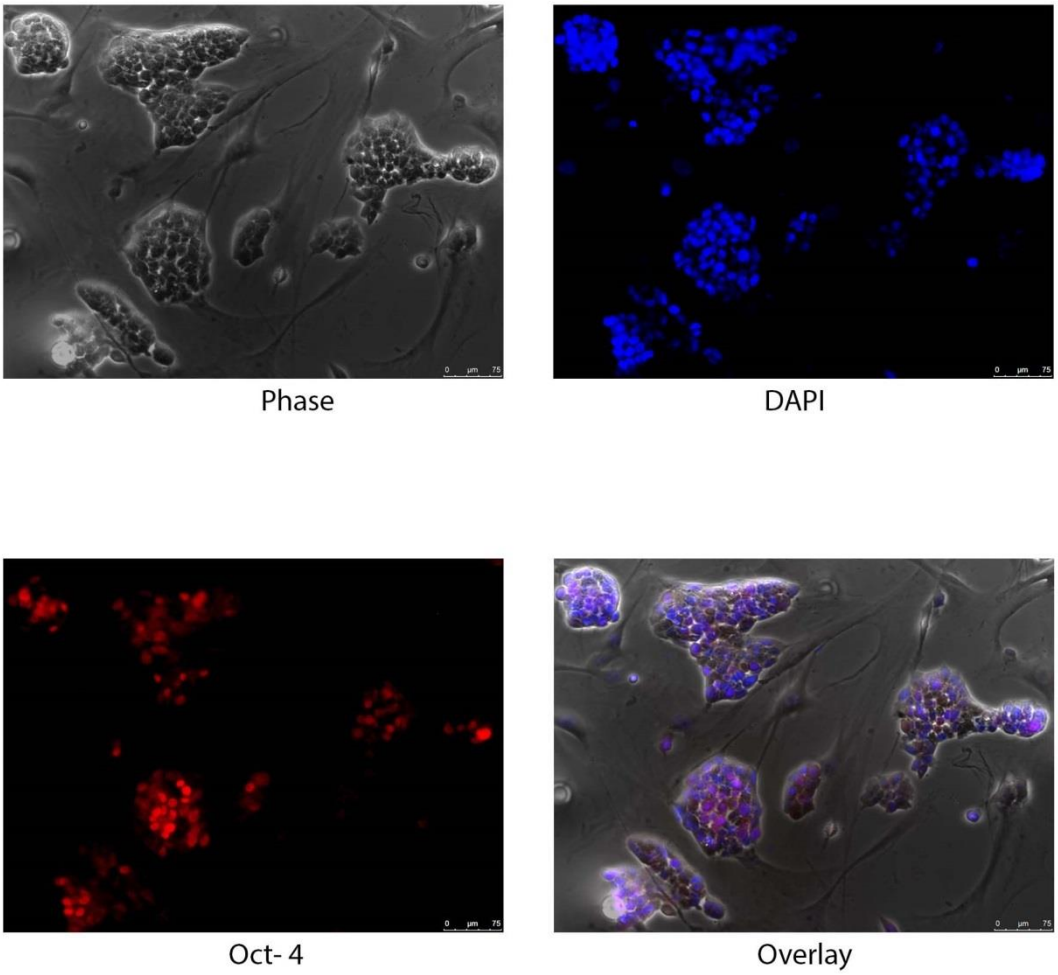


Figure 7: ES cell Pluripotency check using Oct- 4 marker. Left to right is phase, DAPI, Oct- 4 and overlay images respectively.

4.3 ES cell differentiation to yield NPC's

After the ES cell pluripotency was confirmed, the cells were subjected to various stages for differentiation. The ES cells formed 3D aggregates called EB's that differentiated to give rise to cells arising from the 3 germ layers- Endoderm, Ectoderm and Mesoderm.

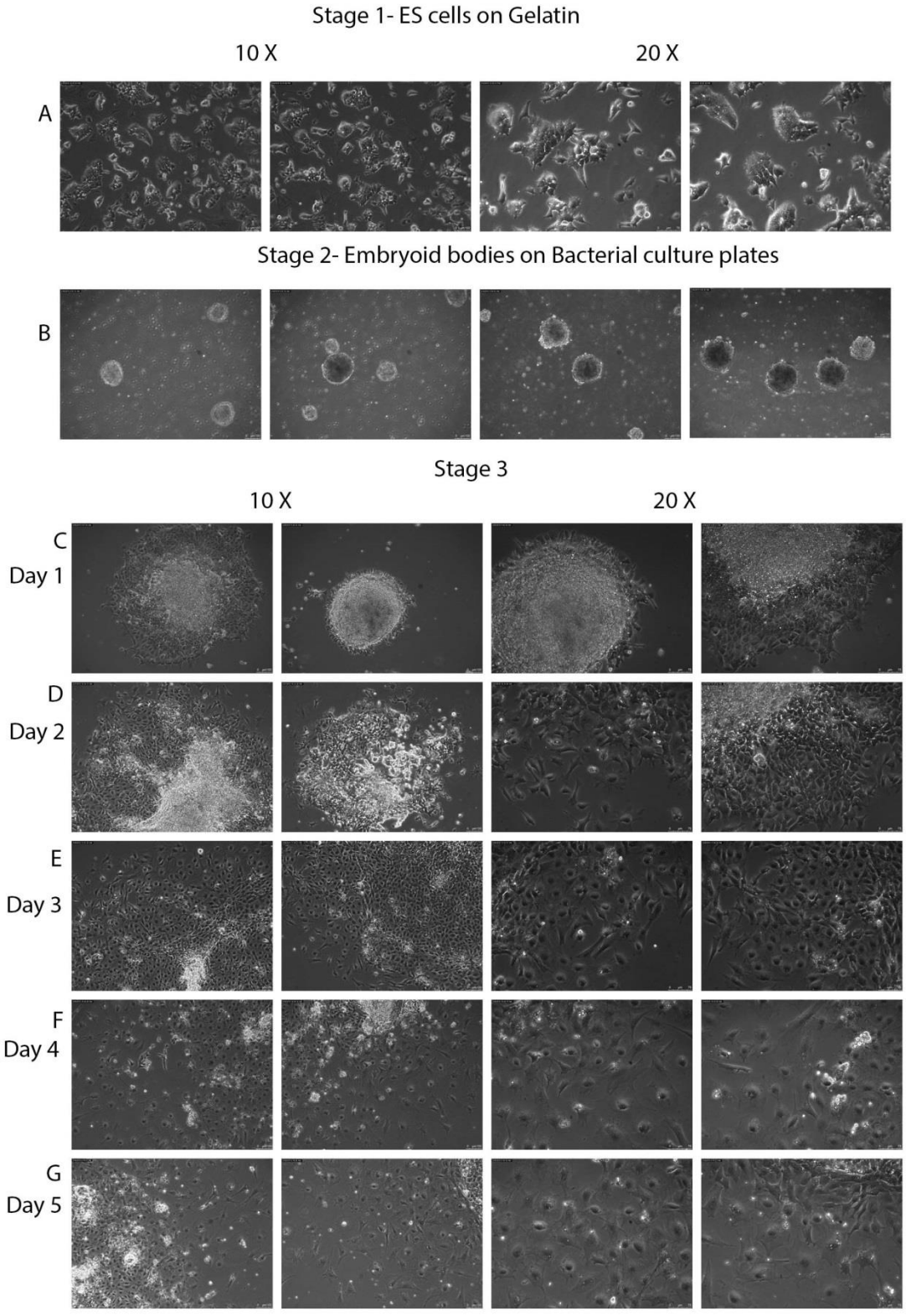


Figure 8: ES cell differentiation to stage 3 cells (a) ES cells were cultured and expanded on gelatin coated dishes (b) Stage 2 is the formation of pluripotent 3D EB's of ES cells on bacterial grade dishes. Cells are cultured for 4 days on bacterial grade dishes and the image shown is EB's at the end of day 4 (c) At the end of day 4, EB's are seeded on regular TC plates and cells radiate out from the 3D structure in ITSFn media. Few cells radiate out at the end of day 1 from EB's. As the day progresses, more and more cells radiate out (c-g).

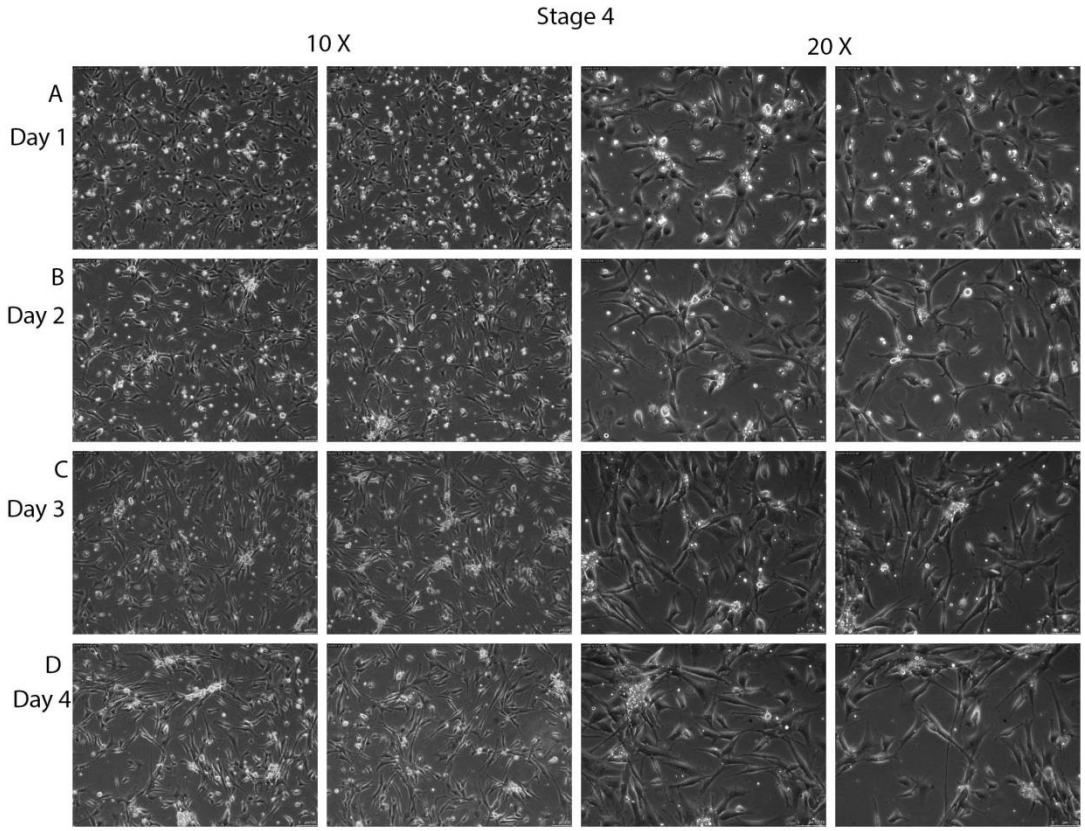


Figure 9: Stage 4 NPC's on Poly- L- Ornithine and fibronectin coated dishes. (A-D) NPC's from stage 3 are selected on the substratum and cultured for 4 days in neural media (D) NPC's are marked by Rosette like structures that are spotted at the end of day 4.

Stage 4 progenitor cells at the end of day 4 were then cultured under different coating conditions listed above for 2 additional days and were stained for rabbit anti- Nestin, goat anti- Sox-2, mouse β III Tubulin/ Tuj1, rabbit anti- GFAP and mouse monoclonal anti- MAP2.

4.4 NPC's under different coating conditions

It was observed that NPC's were obtained and stained positive for Sox-2 and Nestin under all coating conditions. However, the total number of cells observed in each condition was different and this can be seen in the phase images of each condition at Day 2. Maximum positive cells were obtained in PLO and fibronectin coating with

laminin as a growth factor. Number of Sox-2 positive cells was very low in other conditions. Nestin positive cells did not change significantly amongst different coating conditions.

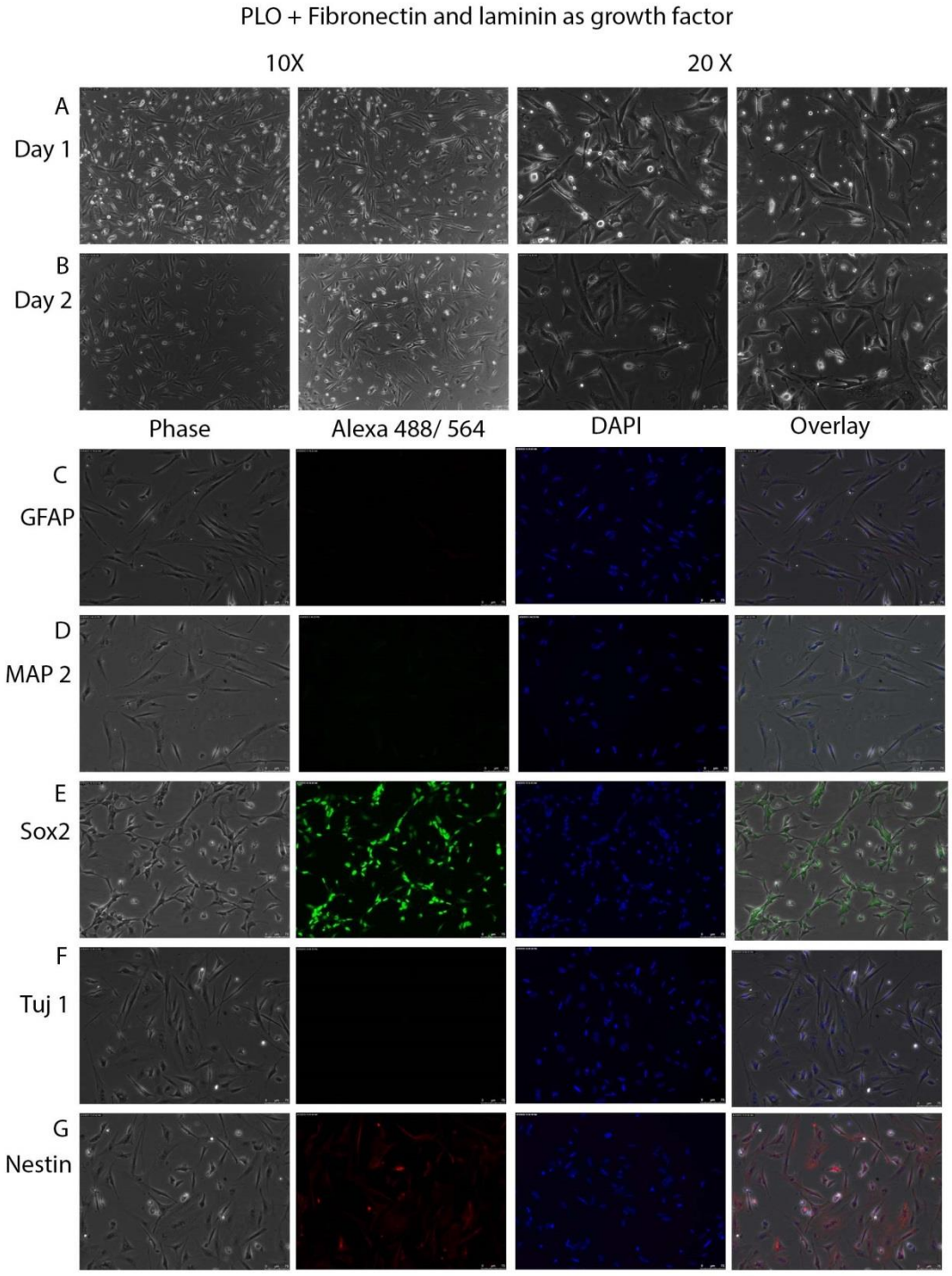


Figure 10: Poly- L- ornithine and fibronectin for coating and laminin as growth factor (A, B) Phase contrast images prior to fixation (C- G) Phase, Alexa 488/ 568, dapi and overlay respectively from left to right. Cells tested positive for Sox2 and Nestin (E, G) and negative for all other glial as well as neural markers (C, D, F).

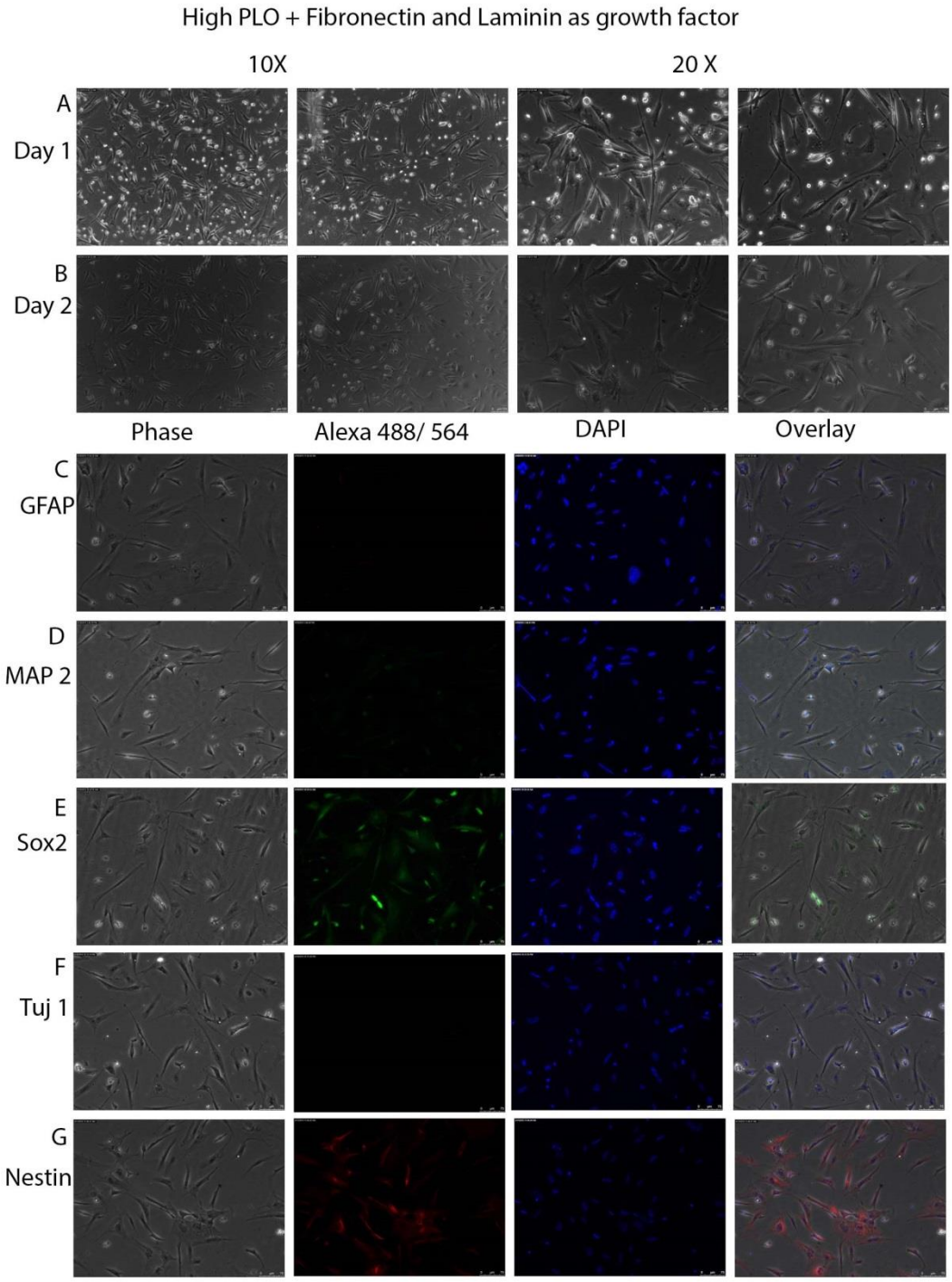


Figure 11: High Poly- L- ornithine and fibronectin for coating and laminin as growth factor (A, B) Phase contrast images prior to fixation (C- G) Phase, Alexa 488/ 568, dapi and overlay respectively from left to right. Cells tested positive for Sox2 and Nestin (E, G) and negative for all other glial as well as neural markers (C, D, F).

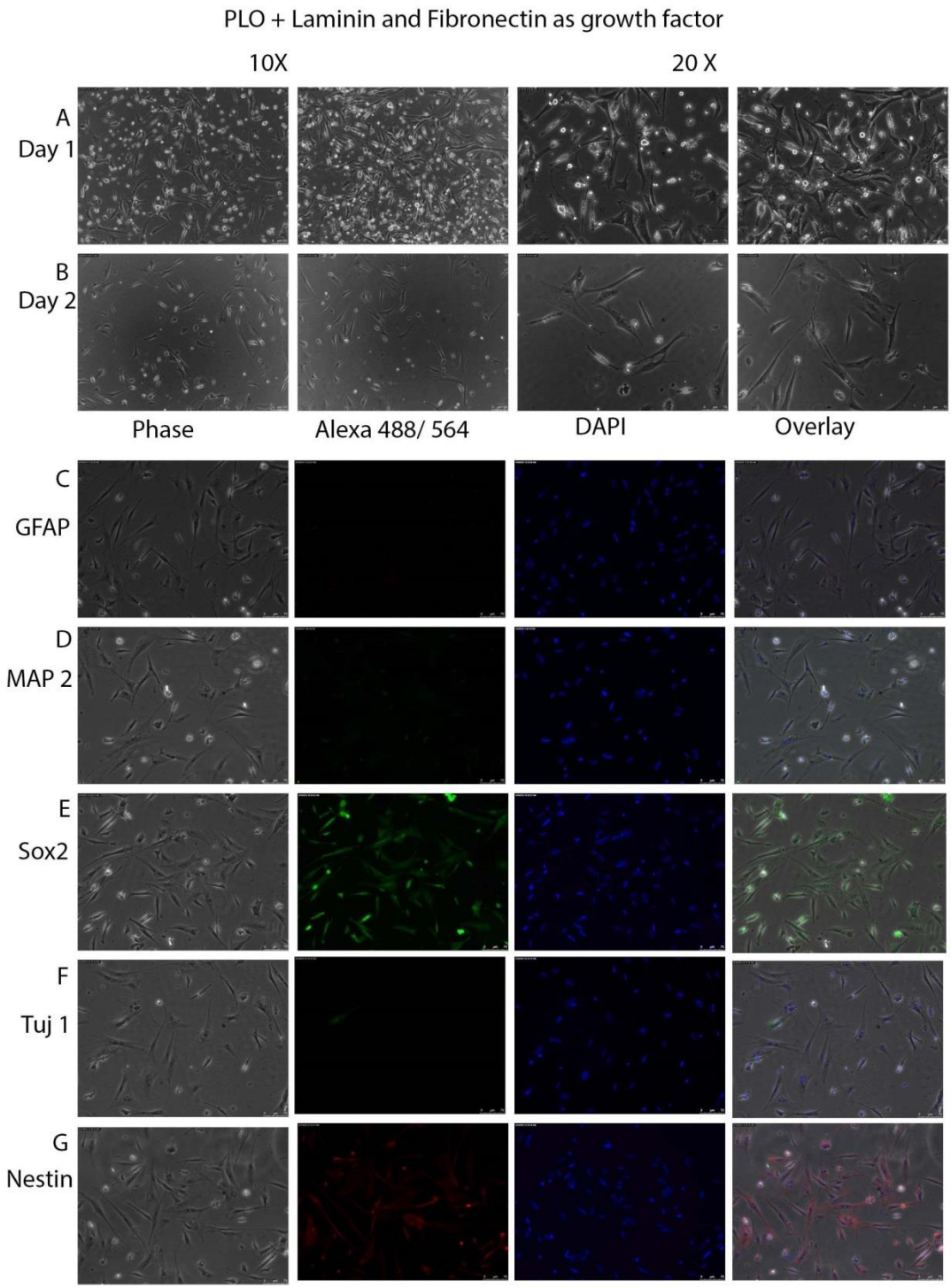


Figure 12: Poly- L- ornithine and laminin for coating and fibronectin as growth factor (A, B) Phase contrast images prior to fixation (C- G) Phase, Alexa 488/ 568, dapi and overlay respectively from left to right. Cells tested positive for Sox2 and Nestin (E, G) and negative for all other glial as well as neural markers (C, D, F).

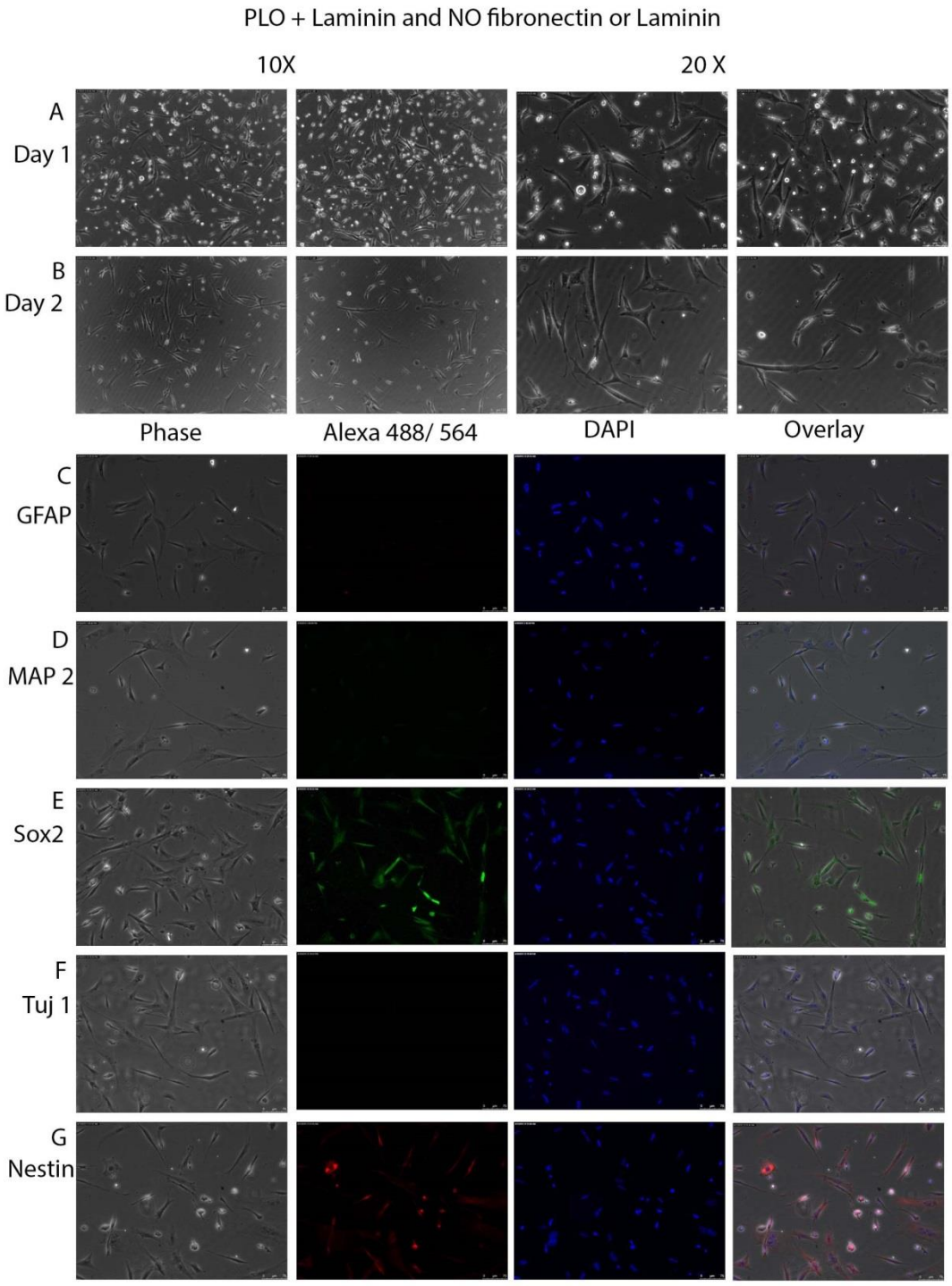


Figure 13: Poly- L- ornithine and laminin for coating and no additional growth factor (A, B) Phase contrast images prior to fixation (C- G) Phase, Alexa 488/ 568, dapi and overlay respectively from left to right. Cells tested positive for Sox2 and Nestin (E, G) and negative for all other glial as well as neural markers (C, D, F).

All the images were processed using a MATLAB code (Appendix E) to count the number of cells positive for NPC markers (Sox-2 and Nestin) and total number of

cells. A ratio of these numbers gave the percentage of cells positive for Sox-2 and Nestin (Figure 14).

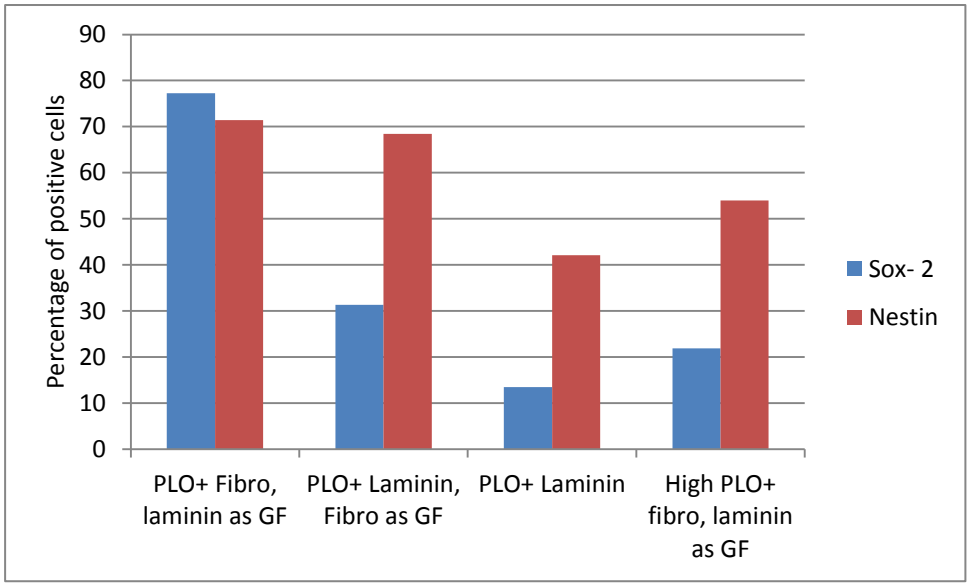


Figure 14: Ratio of Sox-2 and Nestin positive cells to total number of cells for all the four conditions.

4.5 Astrocytes

Astrocytes were obtained when NPC's were seeded at 75,000 cells/ cm^2 and treated for 15 days in media containing serum. Seeding the cells below this density has shown to affect cell survival tremendously. It was observed that the morphology of the cell changes first followed by its transformation to astrocyte type. This was confirmed by staining which showed positive results for GFAP protein. The differentiated astrocytes were compared to primary astrocytes obtained from mice cortex and were maintained in the same media and condition as differentiated astrocytes. This helps in maintaining the same parameters between the cell types.

It was observed that the laminin had a negative effect on the differentiating cells, as very few cells attached to the substratum under laminin conditions. Coating (PLO vs

PLL) did not have any significant effect on the cells. Similarly, no significant difference was observed in 5% serum vs 10% serum condition.

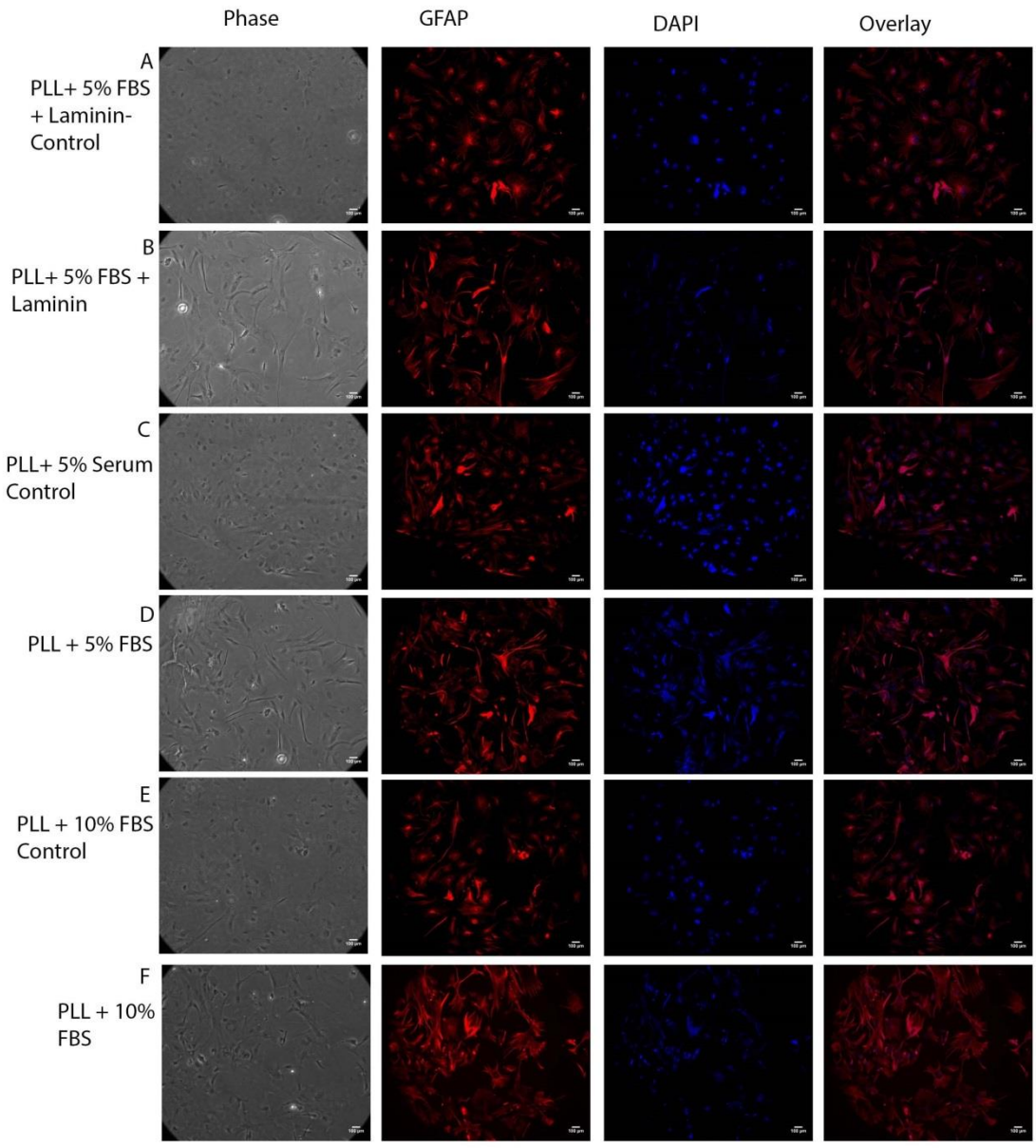


Figure 15: Astrocytes with PLL coating, 5 and 10% serum and laminin conditions stained for GFAP marker. From left to right are phase, GFAP, DAPI and Overlay images.

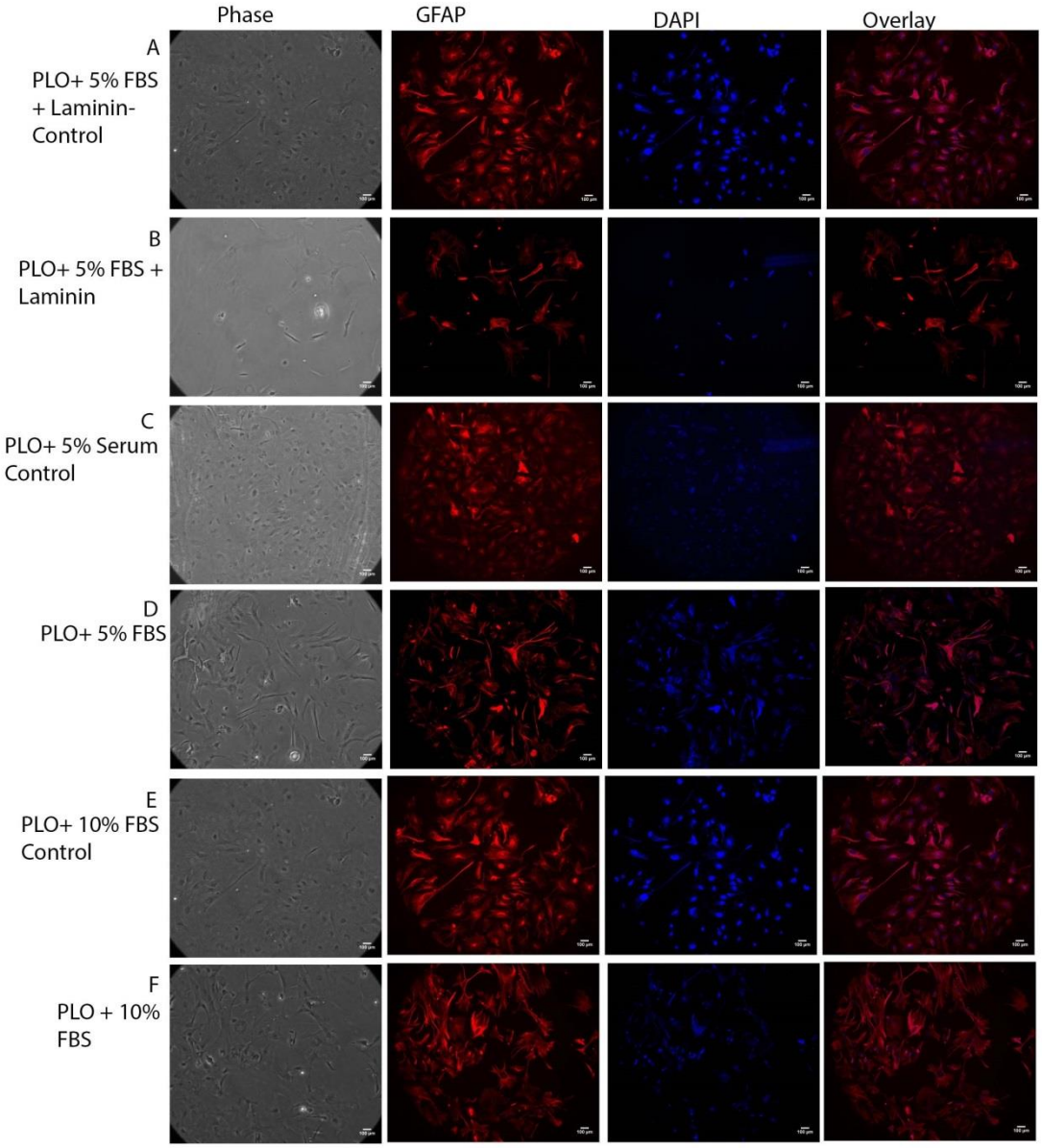


Figure 16: Astrocytes with PLO coating, 5 and 10% serum and laminin conditions stained for GFAP marker. From left to right are phase, GFAP, DAPI and Overlay images.

A MATLAB code was used to determine the number of cells positive for GFAP as compared to total number of cells. The result was unexpected as highest percentage was observed in laminin conditions but this was due to the fact that there was less number of total cells and almost all of those cells were positive for GFAP (Figure 17). Hence we observe highest peak in the graph for laminin conditions.

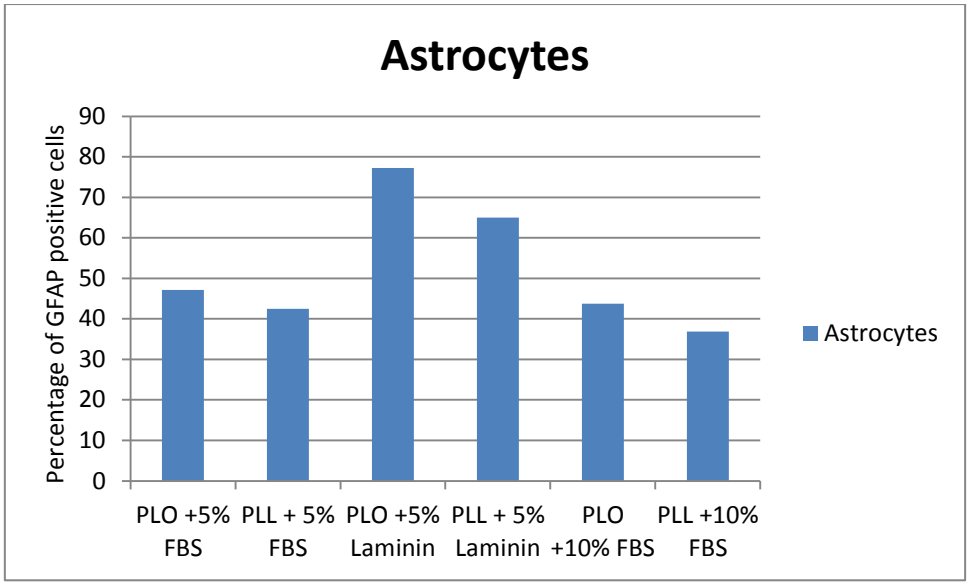


Figure 17: Ratio of GFAP positive cells to total number of cells for all the parameters/ conditions.

5.6 Cortical Neurons

Cortical neurons were generated from monolayer cultures of ES cells 21 days post treatment in the presence of sonic hedgehog inhibitor, cyclopmaine. ESC's followed neurogenesis efficiently leading to the generation of neural progenitors and then neurons. Immense amount of cell death was observed between day 2 to day 12 (Figure 18 and Figure 19). In the later stages, ESC derived neurons displayed patterns of axonal projections that are similar to the neurons of the cortex. This result was confirmed by IF staining. Cyclopamine used in this experiment was to prevent the induction of ventral neural progenitor.

Extensive care was taken to attain single cell suspension at the start of the experiment to prevent excessive crowding. Too many crowed cells on a dish increase the chances of contamination and also interfere with cell proliferation by pushing

them towards senescence. The following 3 parameters were achieved during the experiment:

- ESC health and pluripotency before differentiation (Figure 7)
- ESC density at the time of plating for differentiation
- Efficient dissociation and dispersion of the cells on day 12

24 hours after the passage of ESC's (differentiation day 0) the cells appeared dispersed and were small in size (Figure 18). Later during the differentiation process, neural progenitors were reflected by the formation of a rosette like structure (Figure 19). Neural progenitors acted as control to the cortical neurons during the staining. Neurogenesis started on Day 6 and can be spotted in Figure 18 and it marks the beginning of neural lineage formation.

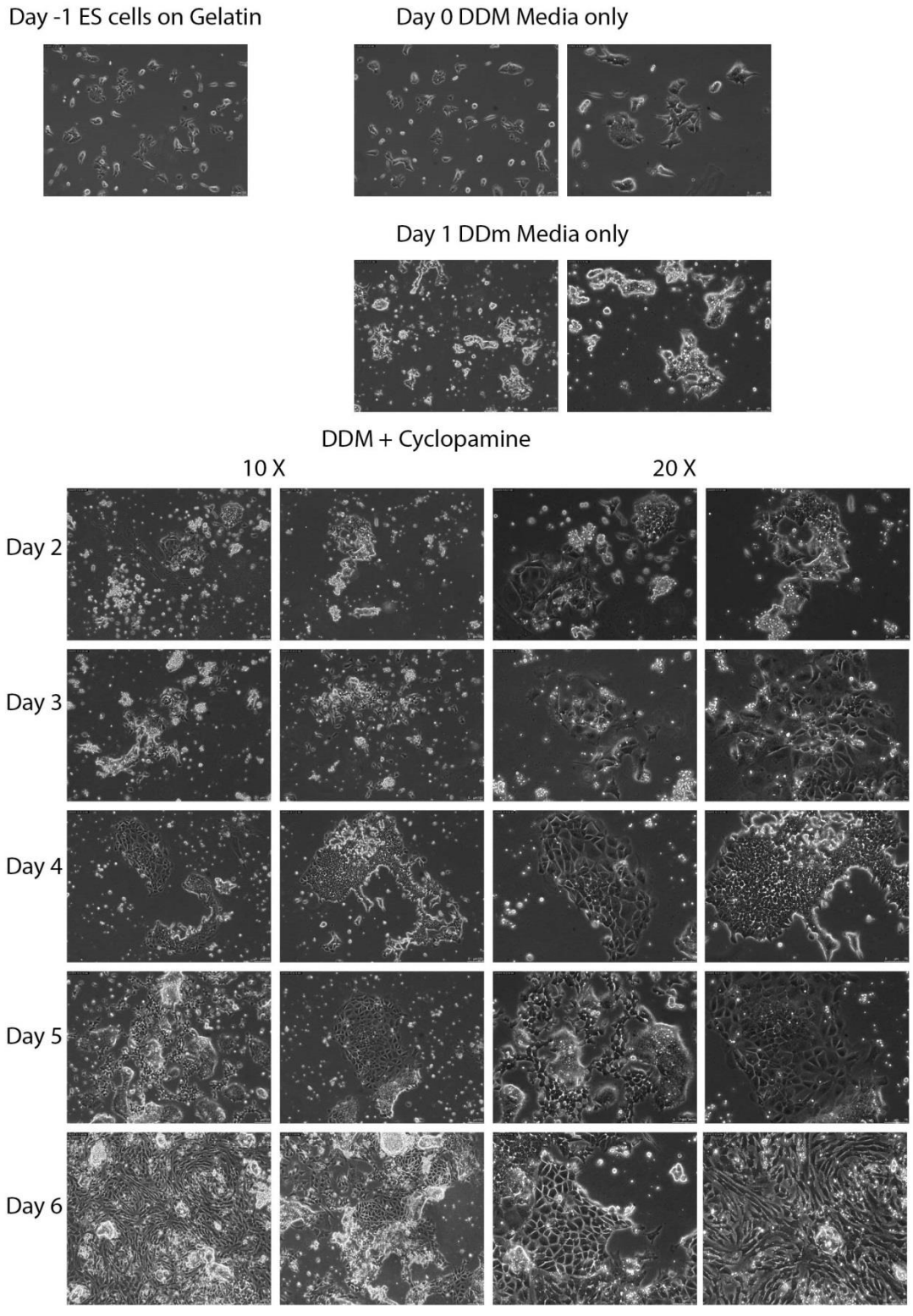


Figure 18: Day -1, ES cells in ES cells media. Day 0 to 2 ES cells treated with DDM media. Day 2 to 9, cells treated with DDM + Cyclopmaine. Day 6 is the start of Neurogenesis. There is immense amount of cell death spotted as the day progresses.

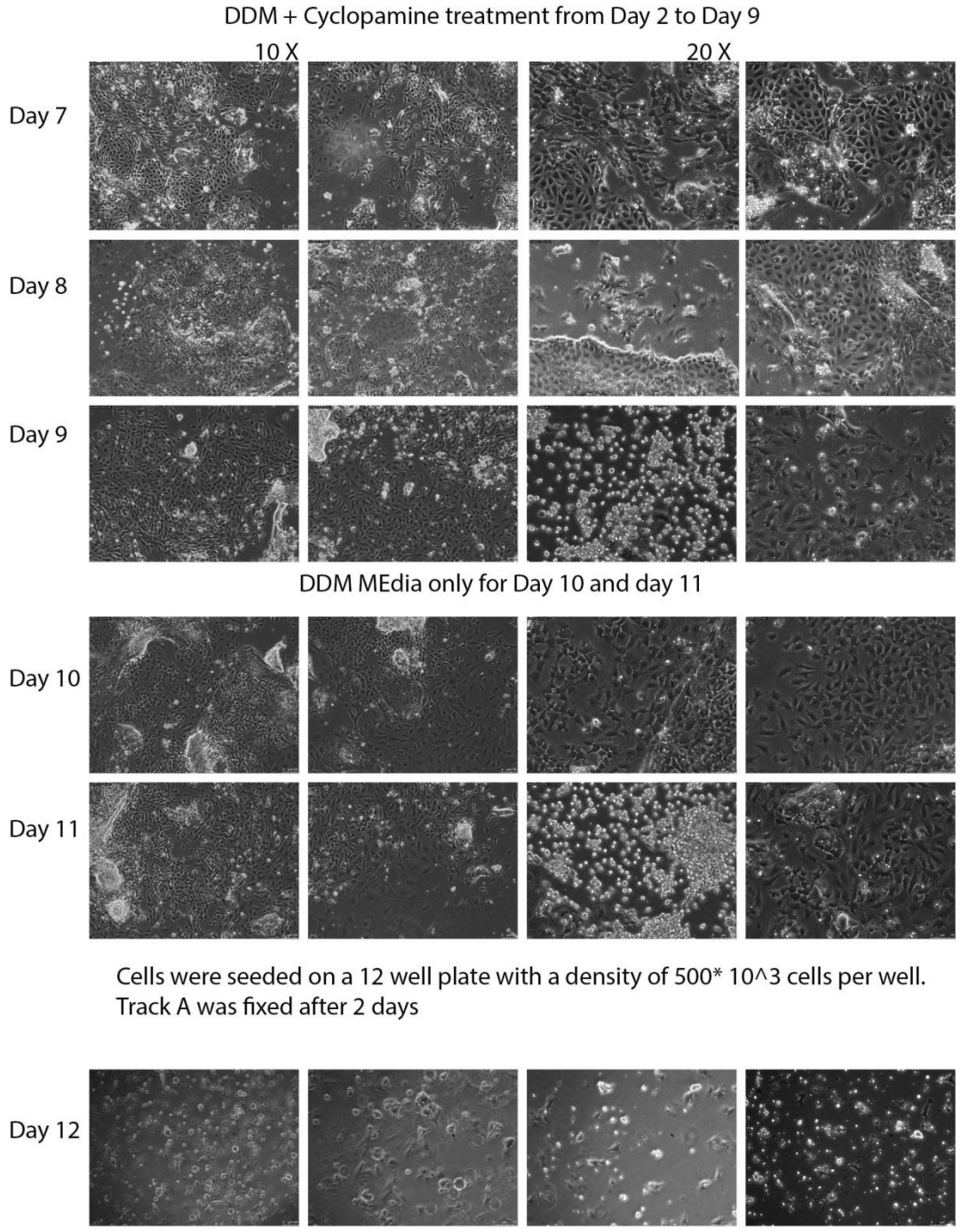


Figure 19: Day 0 to 2 ES cells treated with DDM media. Day 2 to 9 cells treated with DDM + Cyclopmaine. There is immense amount of cell death spotted until day 9 due to the effect of cyclopmaine. On day 12, cells trypsinized and seeded on PLL + Laminin coated coverslips. Track A seeded at 500 * 10^3 cells/ well and fixed after 2 days.

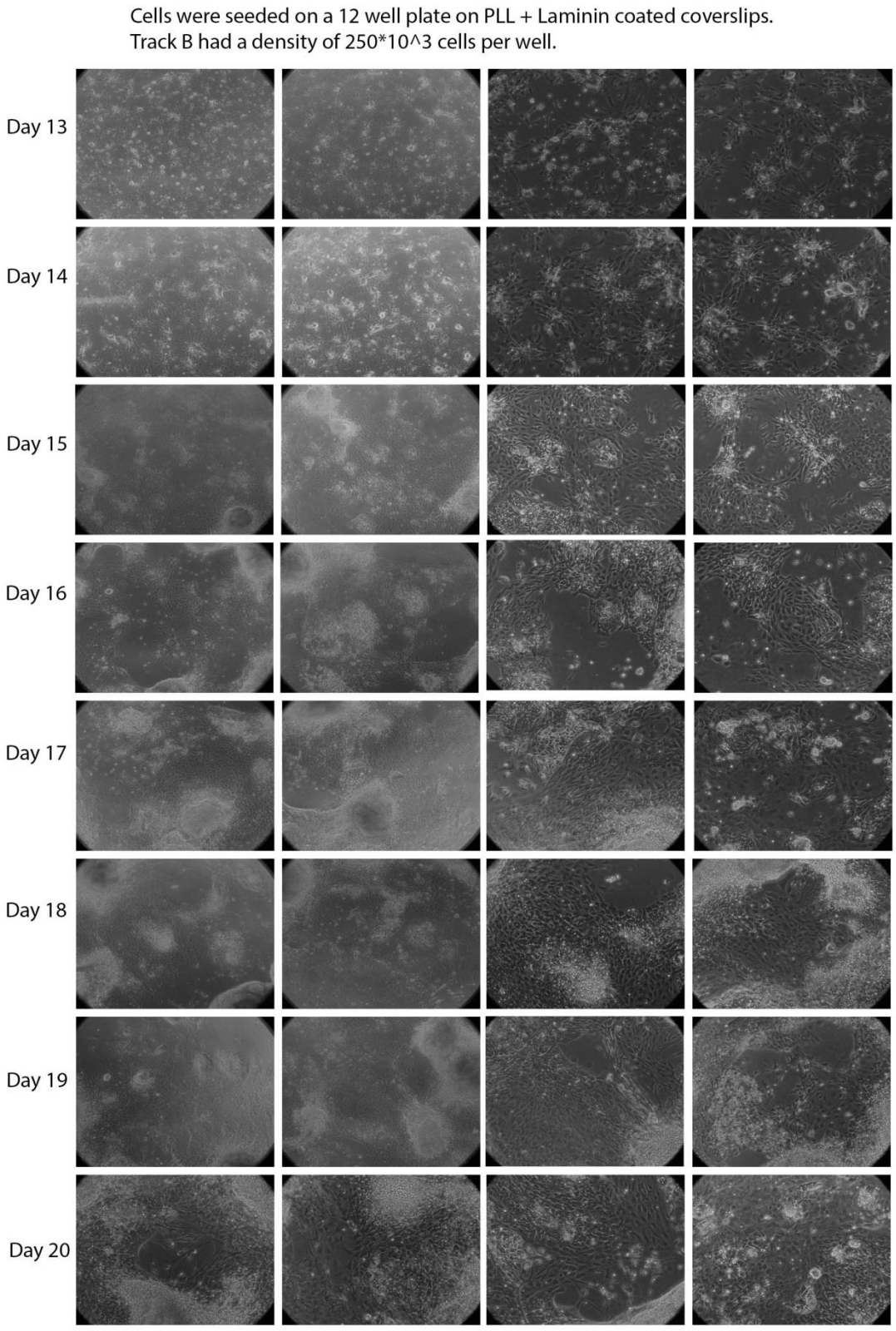
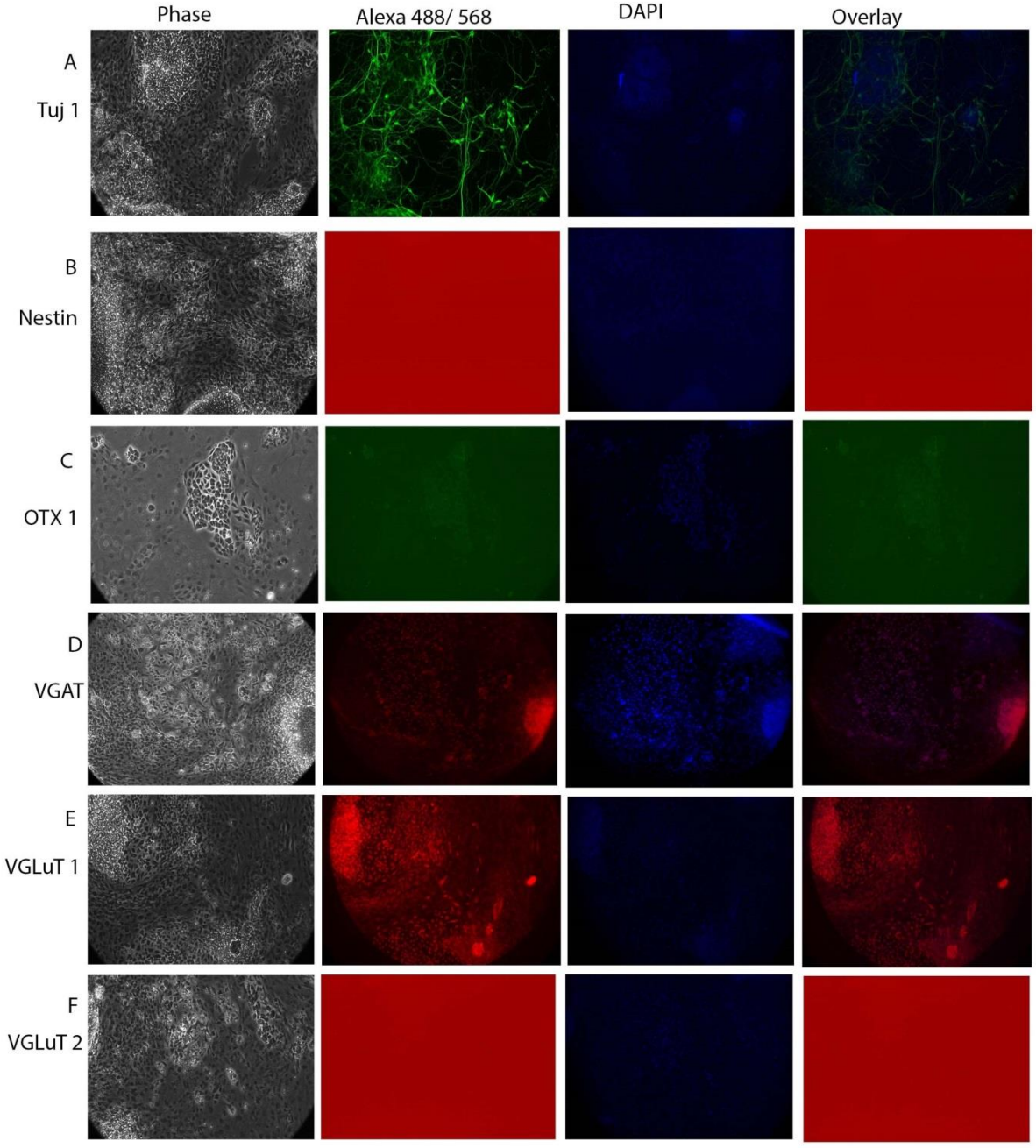


Figure 20: Day 12, cells trypsinized and seeded on PLL + Laminin coated coverslips. Track B was seeded at 250 * 10^3 cells/ well and fixed on day 21.

Cells observed at Day 21 were extremely crowded and there was no room for the cells to divide anymore if kept in culture. Also, there were big cell clusters seen in the dishes showing the cells were aggregating inspite of obtaining a single cell suspension post trypsinization. A few cells were fixed on Day 21, even if they were too overcrowded and were stained for a wide range of markers.



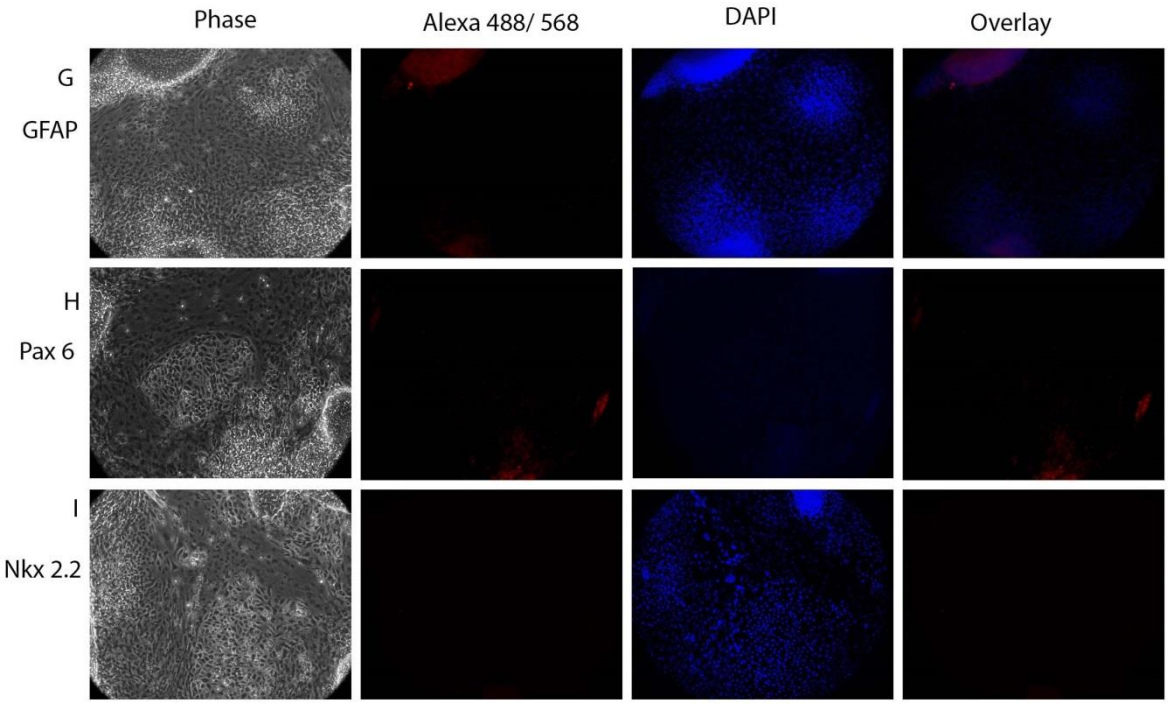


Figure 21: Day 21 cortical cells stained for NPC, Astrocytes and Neurons markers. From left to right Phase, Alexa 488/ 568, DAPI and Overlay images (A) Cells stain positive for Tuj1, neuron marker (B, C and I) Cells stain negative for neural progenitor and midbrain and hindbrain neurons (D, E) Cells stain positive for VGAT and VGLuT1 which are neuron markers (G) few cells stain positive for GFAP marker and (H) very few cells stain positive for pax6, a cortical progenitor marker (I) Cells stain negative for Nkx2.2, ventral progenitors.

It was observed that cells at day 21 were positive for neuronal markers (Tuj1, VGLuT1 and VGAT) and all other glial as well as progenitor markers were negative.

All the images were processed using a MATLAB code to count the number of cells positive for markers and total number of cells. A ratio of these numbers gave the percentage of cells positive for specific markers.

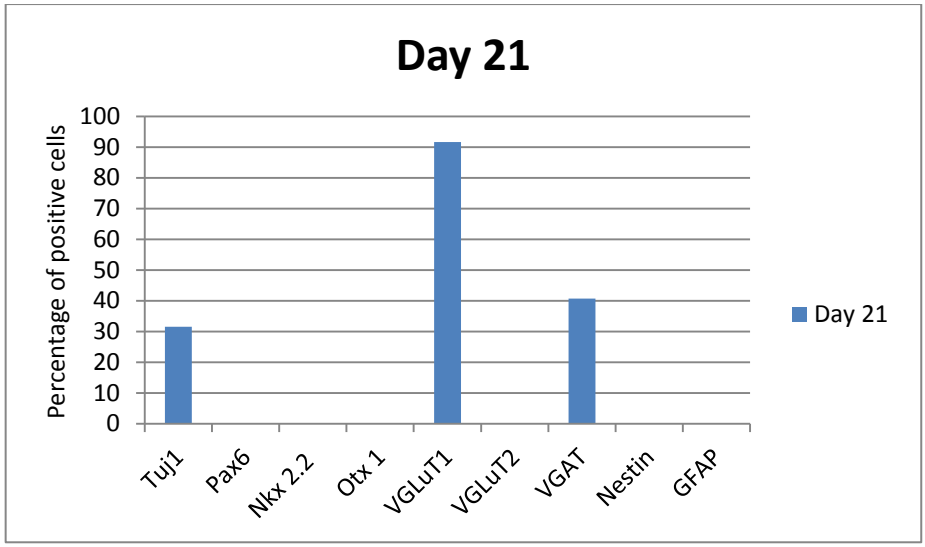


Figure 22: Day 21 cells positive for neuronal markers Tuj1, VGLuT1, VGAT.

Few cells from Day 21 that were not fixed were trypsinized and replated on Poly- L-Lysine and Laminin coated coverslips at different densities and they were then analysed for progenitors or neural markers. Cells analysed for progenitors were fixed 2 days after seeding i.e. day 23 and cells analysed for neuronal markers were kept in culture for 6 additional days and fixed on day 27.

- Track A- Analysis of progenitor cells. Cells were seeded at the density of 125,000 cells/ cm^2.
- Track B- Analysis of neurons. Cells were seeded ay density of 62,500 cells/ cm^2.

Track A- Analysis of progenitors

Cells were kept in culture for 2 more days after trypsinization on day 21. At the end of 2 days, the cells were analysed for the presence of neural progenitor markers.

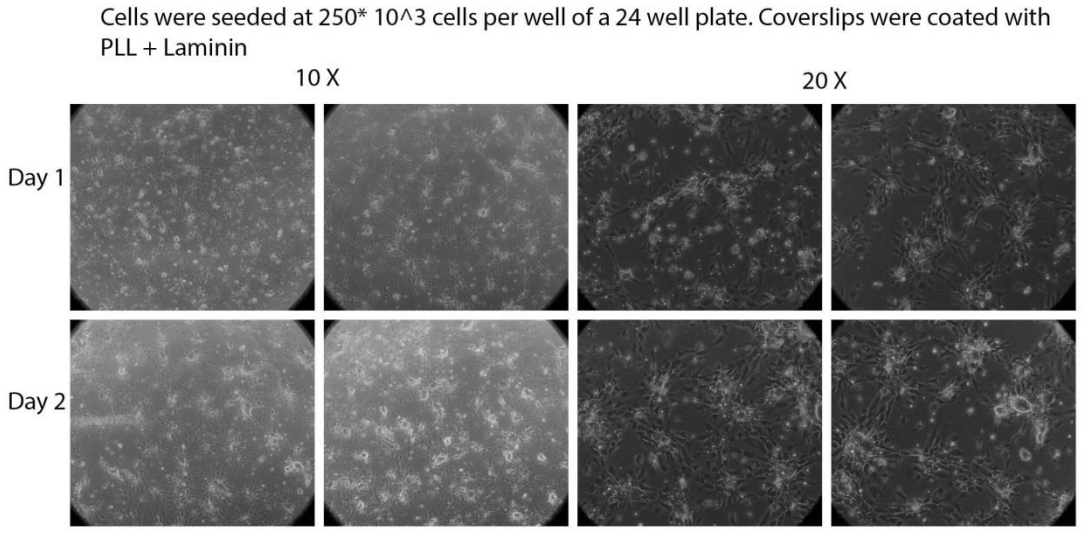
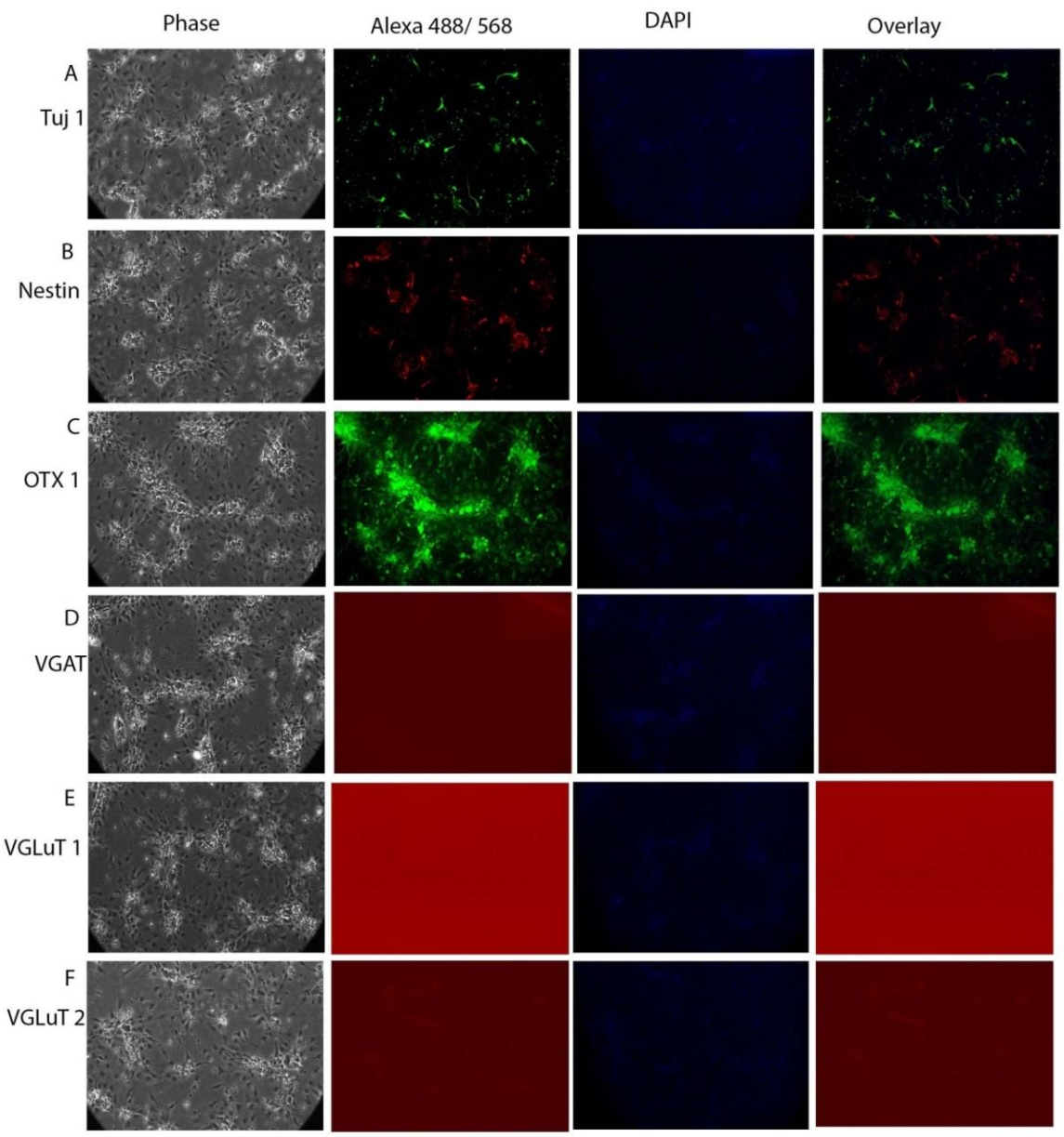


Figure 23: Phase images of day 21 cortical cells for 2 days post trypsinization. The cells were seeded at 250*10^3 cells per well of a 24 well plate pre coated with PLL + Laminin

It was observed that cells at day 23 were positive for progenitor markers (Pax6, Otx1 and Nestin) and all other glial as well as neural markers were negative.


Phase
Alexa 488/ 568
DAPI
Overlay
A
Tuj 1
B
Nestin
C
OTX 1
D
VGAT
E
VGLuT 1
F
VGLuT 2

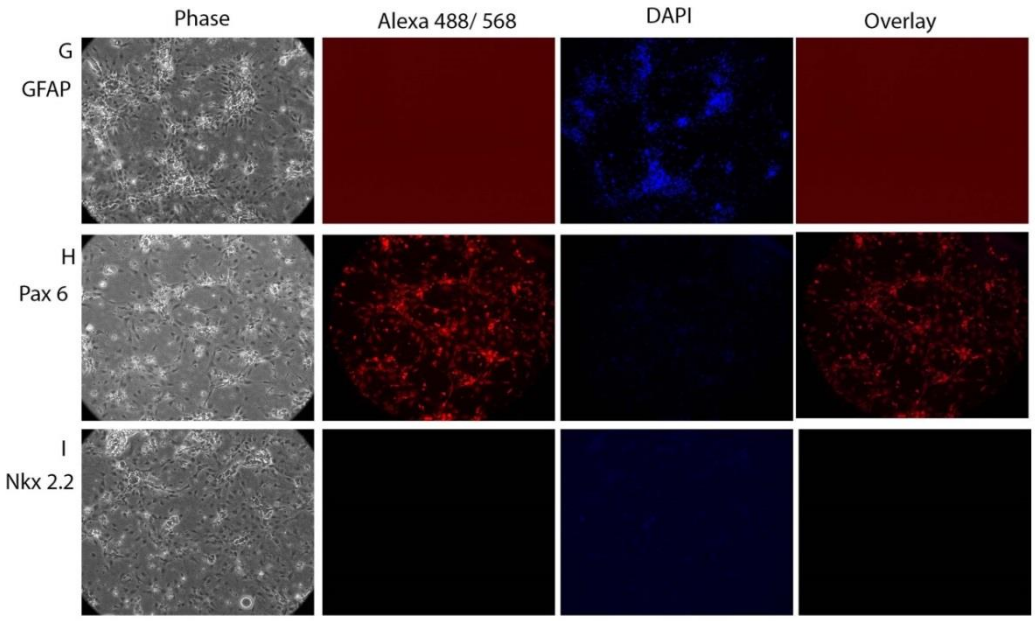


Figure 24: Day 23 cortical progenitor cells stained for NPC, Astrocytes and Neurons markers. From left to right Phase, Alexa 488/ 568, DAPI and Overlay images (A) Very few cells stain positive for Tuj1, neuron marker (B) Cells stain positive for neural progenitor, Nestin (C) Cells stain positive for OTX1, forebrain and midbrain progenitors (D-F) Cells stain negative for VGAT, VGLuT1 and VGLuT2 which are Neuron markers (G) Cells stain negative for GFAP marker and (H) Cells stain positive for pax6, a cortical progenitor marker (I) Cells stain negative for Nkx2.2, ventral progenitors.

The images were processed using a MATLAB code to count the number of cells positive for markers and total number of cells. A ratio of these numbers was plotted on excel to give the percentage of cells positive for progenitor markers.

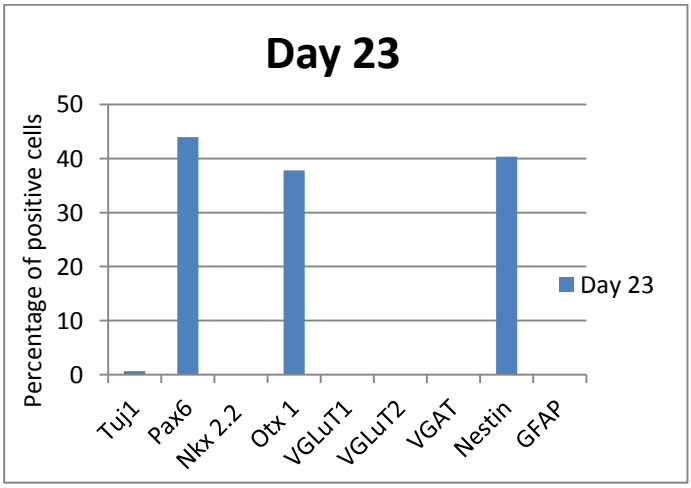


Figure 25: Day 23 cells positive for progenitor markers Pax6, Otx1 and Nestin.

Track B- Analysis of Cortical Neurons

Cells were kept in culture for 6 more days after trypsinization on day 21. At the end of 6 days, the cells were analysed for the presence of neuronal markers.

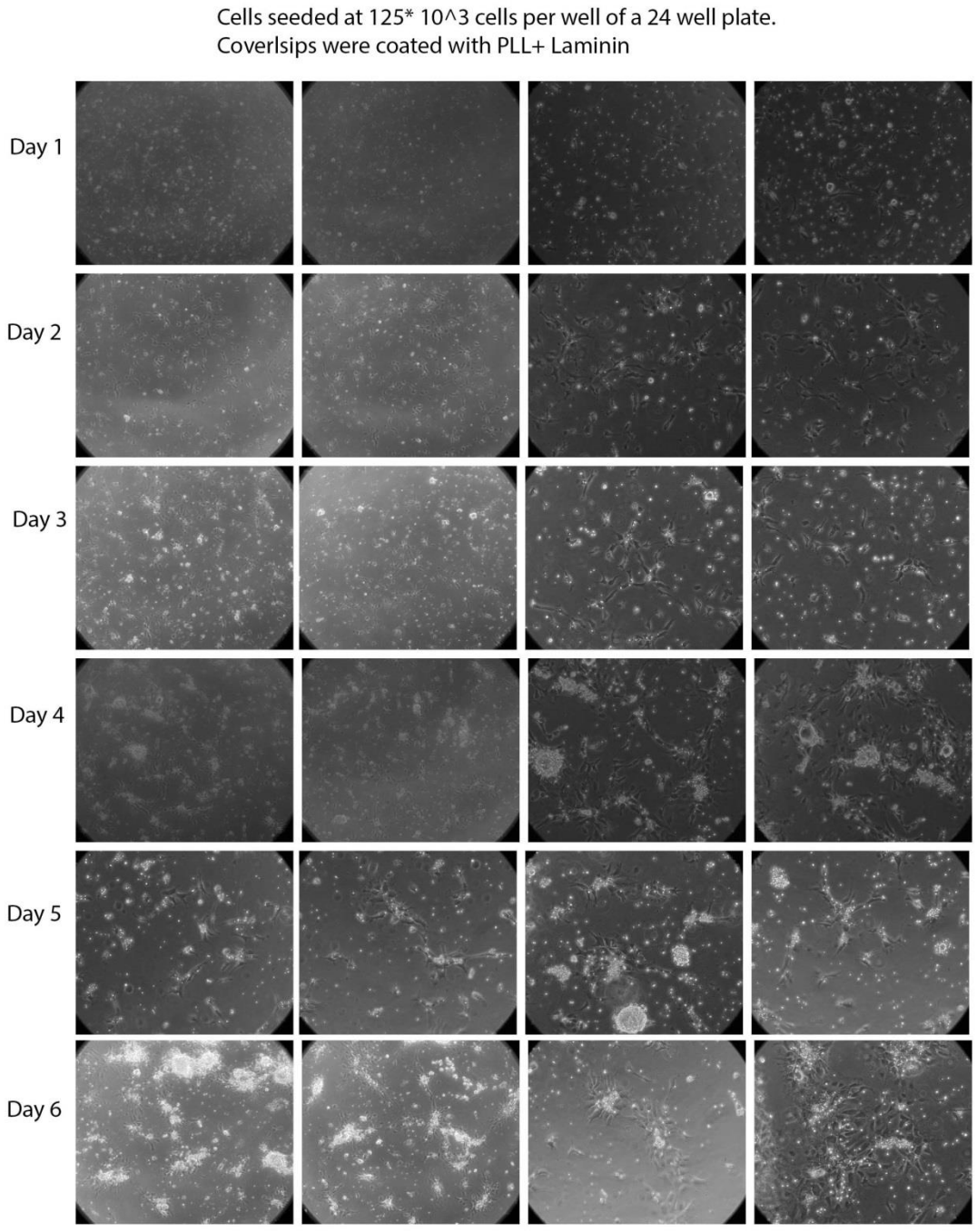
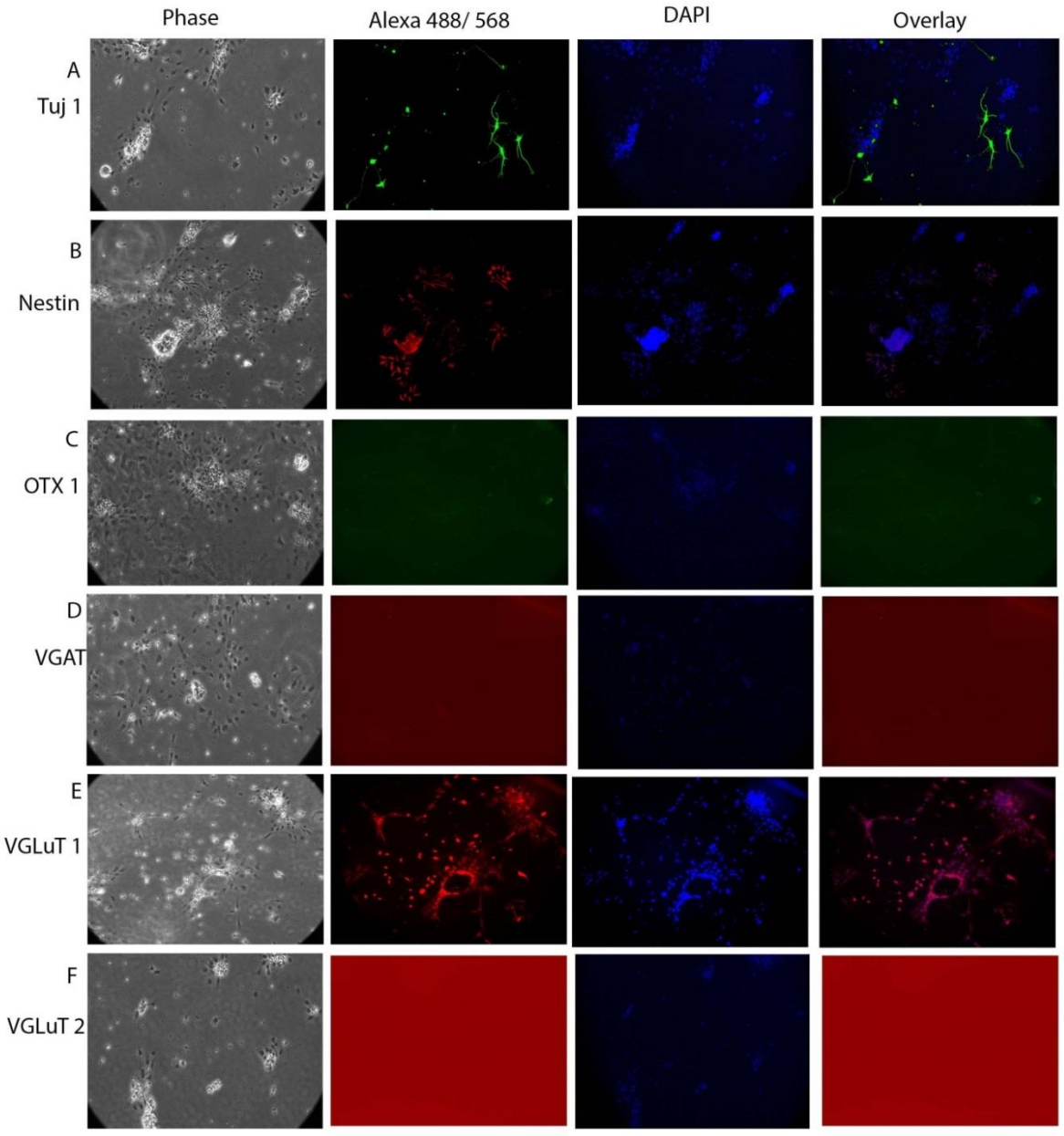


Figure 26: Phase images of day 21 cortical cells for 6 days post trypsinization. The cells were seeded at 125*10^3 cells per well of a 24 well plate pre coated with PLL + Laminin

It was observed that cells at day 27 were positive for neuronal markers (Tuj1, VGLuT1 and VGAT) and all other glial as well as progenitor markers were negative.



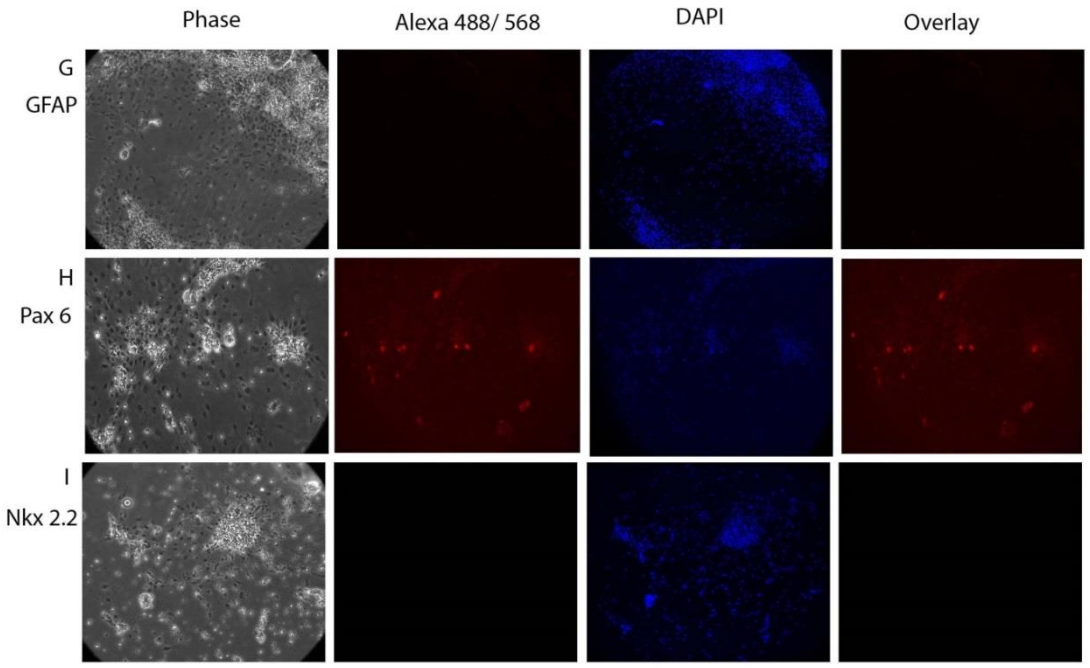


Figure 27: Day 27 cortical cells stained for NPC, Astrocytes and Neurons markers. From left to right Phase, Alexa 488/ 568, DAPI and Overlay images (A, E) Countable cells stain positive for Tuj1 and VGLuT1, neuron marker (B) Very few cells stain positive for neural progenitor, Nestin (C, H) Cells stain negative for OTX1, forebrain and midbrain progenitors and Pax6, cortical progenitor marker (D, F) Cells stain negative for VGAT and VGLuT2 which are Neuron markers (G) Cells stain negative for GFAP marker (I) Cells stain negative for Nkx2.2, ventral progenitors.

The images were processed using a MATLAB code to count the number of cells positive for markers and total number of cells. A ratio of these numbers was plotted on excel to give the percentage of cells positive for progenitor markers.

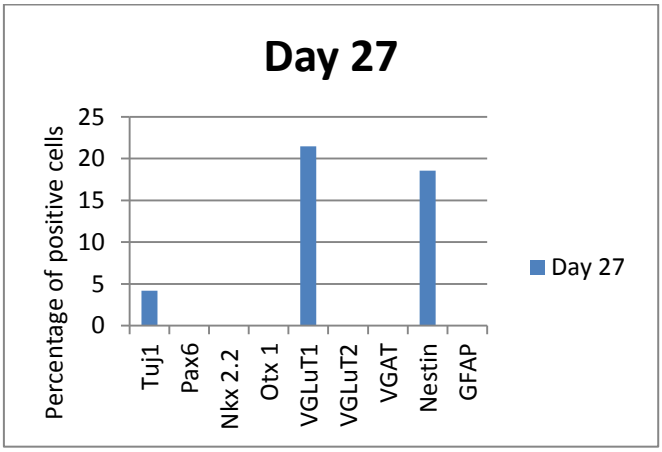


Figure 28: Day 27 cells positive for Neuronal markers Tuj1, VGLuT1 and VGAT.

5. Discussion

5.1 MEF's

MEF's need to be evaluated for their health as they act as feeder layers for ES cells to proliferate. MEF's beyond passage 5 underwent senescence and hence were not used beyond passage number 5. Mitomycin C inhibits the cell division and keeps the cells alive. This means that, the cells are secreting the proteins but are not undergoing any cell divisions. The proteins secreted by the MEF's are used by ES cells for growth and proliferation. Mitomycin C inactivated MEF's at the density of $1.2 * 10^6$ - $2* 10^6$ cells/ 10 cm dish were used as feeder layers for growing ES cells. Seeding below this density and using old plates sitting in the incubator for over 15 days, has shown to compromise the health of ES cells. This is because the cells have been arrested at a particular phase of their cell cycle and would die within a few days due to their incapability to replicate. Old cells show formation of large clumps and eventually are seen floating as clusters in the media.

5.2 ES cell pluripotency

Pluripotency test helps determine the efficiency of ES cells to differentiate into desired cell types. If the ES cells are not pluripotent, they would not differentiate. V6.5 cells tested positive for Oct-4 thereby confirming their ability to differentiate into different types of neurons and astrocytes (Figure 7).

5.3 Differentiation

ES cells were seeded on gelatin and passaged to bacterial grade dishes. Healthy embryoid bodies were obtained which were then subjected to ITSFn media to yield stage 3 cells (Figure 8). The stage 3 cells were then expanded on Poly- L- Ornithine and fibronectin coated dishes for 4 days to give NPC's (figure 9) (Wernig, et al., 2008).

5.4 Neural Progenitor cells

The cells were positive for markers, Nestin and Sox-2 thereby confirming them to be neural progenitors. All other neuron and astrocyte markers were negative (Figure 10- Figure 13). The seeding density of 75,000 cells /cm^2 was used to maintain the necessary cell- cell contact amongst the cells. As a result, NPC's have shown the formation of rosette like structures. Stage 4 NPC's showed best results for the condition where Poly-L- Ornithine and fibronectin were used as coating reagents and laminin was added as growth factor. This can be seen with high cell survival and density (Figure 10). This condition also showed 77.22% of Sox-2 positive cells and 71.43% of Nestin positive cells which was the highest as compared to other conditions (Figure 14). When the concentration of Poly-L-Ornithine was increased, a detrimental effect was seen on the cells with a decrease in cell number in the field of view of the image (Figure 11). 31% of the cells were positive for Sox-2 and 68.42% of the cells were positive for Nestin in this coating condition (Figure 14). Other coating conditions also compromised on cell survival as seen in Figure 12 and Figure 13 and hence they were scraped and not used for further expansion of neural progenitors. They also yielded the lowest number of Sox-2 and Nestin positive cells. This illustrates that fibronectin acts as a better ECM as compared to laminin for

NPC's derived from ES cells. Also, fibronectin plays a role in differentiation and proliferation (Sigma Aldrich) however laminin on the other hand is involved only in differentiation (Sigma Aldrich). In addition, optimal condition for attachment is specific for every cell line and this suggests that differentiated NPC's require different coating conditions as compared to primary NPC's.

5.5 Astrocytes

Both differentiating cells as well as primary cortical astrocytes stained positive for GFAP marker thereby confirming them to be astrocytes (Figure 15 and Figure 16). The various coating, serum concentration and laminin conditions did not serve too much help as all the conditions had a positive signal. This is because the most important factor for differentiation is the time period for which the cells are kept in culture, in my case it is 15 days to obtain astrocytes. Condition containing laminin showed very less cells in the phase contrast view of Figure 15 and Figure 16. This can drive 2 conclusions that either laminin was killing the cells or laminin did not promote cell attachment. In my case, conclusion 1 is scrapped out and the reason for intense cell death is- laminin did not promote cell attachment. Laminin acts as an ECM protein and astrocytes do not require cell- cell contact to promote proliferation.

Also, when a ratio of GFAP positive cells and total number of cells was plotted on MATLAB, the graph showed highest percentage in the conditions involving laminin, 77% and 65% (Figure 17). But what needs to be noted here is the fact that the total number of cells in the field of view was way too less as compared to other conditions (Figure 15 and Figure 16). The total number of cells and GFAP positive cells in other conditions was higher as compared to conditions that contained

laminin. Hence this graph is not very conclusive and what seems to be the best condition by looking at the graph is the worst condition as it did not promote cell attachment. Out of all the different coating conditions that I tried, the most suitable one is PLO+ 5-10% serum as the stage 4 NPC's from which the astrocytes were obtained were also cultured on the same substratum. It is best to not expose the cells to different environment as it affects cell health.

5.5 Cortical Neurons

The cortical experiment was based on a monolayer culture method where ES cells were cultured and neural lineage was obtained by adding neural induction media to the cells. ES cells on day 0 had replicated as more number of cells were spotted than the initial seeding density of 5,000 cells/ cm^2. The cells were kept in DDM media for 2 days before 1uM of cyclopamine was added. A lot of cell death was observed during 8 days of cyclopamine treatment as the ES cells were forced to change their fate to neural lineage. NPC looking cells were spotted on day 6 and this also corresponds to the onset of Neurogenesis (Figure 18).

Cells at Day 21

Cells were too overcrowded with no room for proliferation. This could be because the cells were seeded at a very high density when they were trypsinized for the very first time. Also the ES cell clone, v6.5, has a higher rate of proliferation and the doubling rate for the cells is higher than other ES cell clones. Another possible reason for this observation is inaccuracy to obtain a single cell suspension. It is observed that cells cluster if not suspended in a single cell form and is the major

reason for heterogeneity. In order to obtain a homogeneous population, all the cells need to be well suspended in the media as single cells and should not form clusters in media. Cells stained at day 21 were however positive for neuronal marker Tuj1, VGAT and VGLuT1 and were negative for progenitor markers Nestin, Pax6 and Otx1 (Figure 21). 31% of the cells were positive for Tuj1, 91.6% of the cells for VGLuT1 and 40% of the cells for VGAT (Figure 22). These cells looked heterogeneous to the naked eye but tested positive for neuronal markers which shows that these cells were indeed neurons and neurons grow well at a higher seeding density. However the results are not very concrete as the ratio of Tuj1 and VGLuT1 positive cells are very different. A better way to get concrete results would be to co-stain the cells for Tuj1 and VGLuT1 as that would convey percentage of cells positive for both Tuj1 and VGLuT1.

Cells at Day 23

Cells were positive for NPC markers Nestin, Pax6 and Otx1. Thus all the cells were either mid brain and forebrain progenitors or were cortical progenitors. 44% of the cells were positive for Pax6, 37.82% for Otx1 and 40% for Nestin (Figure 25). During this stage, neuronal markers: Tuj1, VGAT and VGLuT1 were negative (Figure 24). This suggested that passaging on day 21 caused loss of all neurons in the culture and only progenitors managed to survive and adhere on the dishes. This is also because neurons do not divide so passaging them will cause most of them to be lost.

Cells at Day 27

Cells were positive for neuron markers Tuj1 and VGLuT1 which are neuron markers thereby proving that the cells in the dishes were excitatory neurons. 4% of the cells were positive for Tuj1 and 21.46% for VGLuT1 (Figure 28). However, number of cells positive for Tuj1 is less as most of the neurons were lost due to passaging at Day 21. During this stage, progenitor markers: OTX1, Nkx2.2 and Pax6 were negative (Figure 27). There are also 18% cells that tested positive for Nestin (Figure 28) which concludes that the cells are heterogeneous. This suggested that progenitors that were seeded on day 21 eventually were successfully converted to Neurons to some extent at day 27. Also not all progenitors were able to commit themselves to neuronal lineage post passaging and hence we see a decrease in the cells positive for neuronal markers and also a few cells positive for Nestin. The ones that committed themselves were lost due to passaging on day 21.

It is extremely necessary to optimize this experiment so as to obtain a homogeneous population. In the future ES cells would be seeded at even a lower density than 5,000 cells/ cm^2 as v6.5 clone cells have a higher rate of proliferation as compared to other ES cell clones. This can be seen by the cells doubling on day 0 and day 1 in DDM media. Also the seeding density at day 12 should be lowered than 62,500 cells/ cm^2 to avoid excessive clumping on day 21. Lowering the seeding density and obtaining a single cell suspension would definitely lead to a homogeneous population.

6 Future Plans

Future direction is to try genetic reprogramming for 3 simple reasons:

- The time period involved in genetic reprogramming is way less as compared to the time involved in differentiation using growth factors and induction media.
- Pure populations of cells are obtained
- There is no spontaneous differentiation. Cells when undergo differentiation have the tendency to spontaneously differentiate thereby yielding a heterogeneous population. In case of genetic reprogramming this problem is avoided.

Secondly, I would like to make large cell pellets each containing 10 million cells of different cell types and study the Chromatin architecture of each of them. The various cells types would include; differentiated NPC's, primary NPC's, differentiated astrocytes, primary astrocytes and cortical neurons. I would be very curious to see the difference in genome folding in these different cell types and compare them to primary cell types by analysing PCA plots. I would also be interested in seeing how the chromatin architecture changes in diseased models as compared to healthy cell models.

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Appendix A: Protocol to obtain MEF feeder layers

Dissection of Mice

1. Sterilise all the dissection instruments (Scalpel, blade, scissors, forceps, tweezers) in autoclave
2. Sacrifice a pregnant mouse at Day 13 or 14 post coitum by dissecting the uterine horns.

Preparation of Gelatin coated tissue culture plates

3. Ready to use 0.1% gelatin solution is used to make tissue culture plate.
4. 8 ml of gelatin is sufficient for covering an entire 100 mm dish and left for incubation in the tissue culture hood for minimum 30 minutes.
5. After 30 minutes, aspirate out all the gelatin and leave the plates open for a few minutes for drying. Use these plates as feeder layers for culturing MEF's.

Isolation of Embryonic Fibroblasts

6. Rinse the Uterine horn by dipping once in a 50 ml centrifuge tube containing 70% v/v ethanol. Incubate it for 5 min in a 50 ml centrifuge tube containing PBS without ions.
7. Under aseptic conditions separate each embryo from its placenta and embryonic sac.
8. After obtaining individual embryos, cut out the head and other non-visceral organs (they are red organs in the embryo).
9. Rinse the remaining pieces of embryo in PBS and collect them in a 50 ml conical flask.
10. After collecting all the pieces of embryo, transfer them in a clean petri dish.

11. Mince the embryos using a sterile blade (Catalogue no- 12-460-108 Militex INC) and chop them into small pieces. The mincing must be as fine as possible so that they can be pipetted.

12. Add 2 ml of 0.05 trypsin/ EDTA (Invitrogen 25300054) and 100Kunitz units of DNase I to the minced embryo in the petri dish and incubate for 5 minutes in incubator.

13. Transfer the contents to a 15 or 50 ml centrifuge tube and incubate for 15 min in a water bath at 37 °C. During the incubation, after every 5 minutes dissociate the cells by pipetting them up and down. Hence, in the time frame of 15 minutes during incubation, we would have to dissociate the cells 2- 3 times.

14. Inactivate the trypsin by adding 2 to 3 ml of MEF media to the centrifuge tube and centrifuge it at 1000 rpm or 300g for 5 minutes.

15. Aspirate out the supernatant without disturbing the pellet and resuspend the pellet with 2 to 3 ml warm MEF medium.

16. Seed the cells onto gelatin coated tissue dishes and expand the cells in the incubator.

17. The cells become confluent in 2 days and this is when we split the cells as well as freeze some cells for future use.

Note: It is great if MEF's are frozen down at an earlier passage number. When thawed, they can be expanded upto P5 and used as feeder layers. Beyond P5, the cells become senescent.

Appendix B: Protocol to obtain NPC's

This protocol was based on 'Neurons derived from reprogrammed fibroblasts functionally integrate into the fetal brain and improve symptoms of rats with Parkinson's disease' paper (Wernig, et al., 2008).

Stage 1: Thaw and expand ES cells

1. Thaw ES cells on a 100mm dish with $2*10^6$ mitomycin inactivated MEF's. Use ES media + LIF to expand ES cells.

ES media composition:

DMEM, high glucose

FBS: 15% v/v

Penicillin- Streptomycin: 1% v/v

NEAA: 1% v/v

L- Glutamine: 1% v/v

BME: 1% v/v

2. Passage them on gelatin (on 100mm dish) when the colonies are at risk of touching each other. This would approximately be on day 2 after thawing.

3. When colonies are at a risk of touching on gelatin coated plates, proceed to stage 2 to make EB's. Count cells and seed $2*10^6$ cells on a bacterial grade 100 mm dish. Before proceeding to stage 2, it is necessary to have only ES cells in culture. All MEF's at this point of time would be lost due to passaging on gelatin.

Stage 2: Make EB's and plate

4. EB's are allowed to grow on a rotary set at 40 rpm in ES cell media without LIF for 3 to 4 days. All the cells clump together and form 3D cell aggregates. Change media every 2 days.

5. Change of media is done by collecting all the media with EB's in a 15 ml conical flask. Let the EB's settle for 5 min and then aspirate out the old media carefully. All new ES media without LIF to the conical tube and gently pipette twice using a Pasteur pipette. Transfer all the media containing the EB's on a new 100 mm bacterial grade plate.

6. After 4 days, collect all the media in a conical flask and let the EB's settle. Aspirate out the old media and add 20 ml of new ES media without LIF to the EB's and then serially dilute the contents on regular TC dishes.

7. Add up the volume in each plate and leave the EB's with ES media without LIF in the incubator overnight for attachment.

Stage 3: ITSFn for 7 to 8 days to select NPC's

ITSFn Media composition:

DMEM/ F- 12

Apotransferrin (50ug/ml)

ITS supplement (0.01 mg/ml)

Fibronectin (2.5ug/ml)

Penn- Strep (1% final volume)

8. After overnight incubation, aspirate ES cell media and add 10 ml ITSFn media to each plate.

9. Change the media every other day.

10. After 5-6 days, trypsinize the cells and expand the NPC's (stage 4).

Stage 4: Expand NPC's for 4 days

11. Use Fibronectin and Poly-L-ornithine coated plates for NPC expansion.

12. Poly-L-Ornithine (15ug/ml) is reconstituted with cell culture grade water and left on the plates for 1 hour at room temperature in the tissue culture hood. Wash 3X with water.

13. Reconstitue fibronectin (1ug/ml) with cell culture grade water and incubate the plates for 1 hour in the tissue culture hood.

14. Seed NPC's at 75,000 cells/ cm^2 and use NPC media for expanding cells. Add growth factors everyday to the cells.

Stage 4 NPC expansion media composition:

DMEM/ F-12

Apotransferrin (100ug/ml)

Insulin (5ug/ml)

Sodium Selenite (30nM)

Putrescine (100nM)

Penn- Strep- 1% of final volume

Mouse laminin- 1ug/ml

bFGF 2 (10ng/ml)

EGF (20 ng/ml)

Note: Laminin, EGF and bFGF2 is added to the media after filtration.

15. The cells were characterised with Immunofluorescence staining using a wide variety of markers.

Appendix C: Protocol to obtain Astrocytes

1) Trypsinize the stage 4 NPC's and seed them on (15ug/ml) PLO coated plates at the density of 75,000 cells/ cm^2.

2) Culture the cells for 15 days in DMEM high glucose, 5-10% serum and 1% Penicillin-Streptomycin for 15 days in an incubator at 37° C.

3) Characterise the cells with immunofluorescence staining and observe if the cells are positive for GFAP.

Appendix D: Protocol to obtain cortical neurons

This protocol was based on 'Generation of cortical neurons from mouse embryonic stem cells' paper (Gaspard, Bouschet, Herpoel, Naeije, Ameele, & Vanderhaeghen, 2009).

ESC thawing

1. Remove a vial of frozen ESCs from the liquid nitrogen tank. Place the vial in the 37 °C water bath until the cells begin to thaw.
2. Add 1 ml of warmed ES media to the vial, resuspend the cells and transfer the cell suspension to a 15-ml tube. Add 8 ml of warmed ES media and resuspend again.
3. Centrifuge for 3 min at 290g, RT. Discard the supernatant by aspiration.
4. Resuspend the cells with 10 ml of ES medium. Incubate the cells in an incubator at 37 °C, 5% CO_2.

ESC expansion

6. Discard the medium everyday and replace with 10 ml of ES medium until the cells become 60% confluent. This should occur 2–3 days after thawing.

ESC passage

7. One hour before the passage of ESCs, replace the media with 10 ml of fresh ES medium to get rid of dead cells and debris.
8. Aspirate the medium and rinse the cells with 10 ml of PBS, aspirate and add 3 ml of 0.05% (wt/ v) trypsin/ 0.5mM EDTA per dish.
9. Incubate for 5 min at 37 °C in the incubator.

10. Pipette 3–4 times with a 1-ml tip and inactivate the trypsin by adding 6-7 ml of ES media. Resuspend the cells by pipetting up and down with a 1-ml filter tip several times.

11. Transfer the cells to a 15-ml tube and centrifuge for 3 min at 290g, RT. Aspirate the supernatant.

12. Add 2 ml of ES medium to the cell pellet and resuspend into a single-cell suspension using a Pasteur pipette. Add 8 ml of ES medium to the 2-ml cell suspension and resuspend well by pipetting up and down several times.

13. Plate the cells on 100mm gelatin coated dish and let the cells attach overnight.

14. On next day, trypsinize the cells and count. Adjust the concentration to 30,000 cells/ ml with ES medium.

15. Transfer 5 ml of suspension (i.e., 150,000 cells) onto a 60-mm gelatin-coated dish, providing a plating density of approx. 5,000 cells/ cm^2. Culture the cells for 12 days in an incubator at 37 °C, 5% CO2.

Early differentiation

17. Remove the ES medium and rinse the dishes with warm (37 °C) PBS. Add DDM media and this is considered as differentiation day 0.

18. After 2 days (differentiation day 2), replace the medium with DDM supplemented with cyclopamine (1uM).

DDM+ Cyclopamine:

Add Cyclopamine to DDM just before warming (1000 times dilution) i.e. add 500ul to 500ml DDM media.

19. Culture the cells for 48 hrs in an incubator at 37 °C, 5% CO_2 and repeat step 18 three times until differentiation day 10.

20. On differentiation day 10, replace the medium with DDM only (no cyclopamine).

ESC-derived neural progenitor's dissociation and late differentiation

21. At differentiation day 12, coat 15-mm coverslips (in 24 well plates) with poly-L-lysine and laminin and leave them for 2 hours at room temperature.

22. One hour before passaging the cells, remove the medium and add fresh N2/B27 (5 ml media/ 60-mm dish).

23. Rinse the cells with PBS, and trypsinize with 1 ml of 0.05% (wt/v) trypsin/ 0.5mM EDTA per dish. Incubate in an incubator at 37 °C until the cells.

24. Pipette the cells up and down with a 1-ml filter tip.

25. Inactivate the trypsin by adding 4 ml of 10% (v/v) FBS (in PBS) and resuspend the cells by pipetting up and down 4–5 times with filter tips and then with a Pasteur pipette.

26. Transfer to a 15-ml tube and centrifuge for 3 min at 290g, RT. Aspirate the supernatant.

27. Resuspend the cell pellet in 1 ml of N2/B27 by pipetting up and down successively with filter tips and Pasteur pipette. Add 4 ml of N2/B27 to the 1-ml cell suspension and resuspend the cells well by pipetting 4–5 times.

Note: Usually, resuspending the pellet in 1 ml medium is too difficult to count. Hence I resuspended in higher volume (10 ml or more) to get a total of approx. 5 million cells/ 60mm plate. Resuspending the cells to a single cell suspension is very crucial so as to obtain a homogeneous population.

28. Count the number of cells. Usually, approximately 8- 10 million cells per 60-mm dish are obtained.

29. Cells were seeded onto coverslips at different densities depending on the downstream analysis.

Option A- for the analysis of progenitors at differentiation day 21,

Option B- for analysis of neurons at differentiation day 21

However in my case analysis for option A was done on day 23 and analysis for option B was done on day 21 and day 27.

(A) NPC analysis on differentiation day 23

(i) Seed 250* 10^3 cells/ well of a 24 well plate containing coated coverslips in N2/B27 medium.

(ii) Culture the cells for 2 additional days in an incubator at 37 °C, 5% CO_2.

(B) Neuronal analysis at differentiation day 21 and day 27

(i) Seed 125* 10^3 cells/ well of a 24 well plate containing coated coverslips in N2/B27 medium.

(ii) Culture the cells for 9 additional days in an incubator at 37 °C, 5% CO_2.

(iii) Change the N2/B27 medium every 2 days until stopping the experiment for analysis. (stop cultures at differentiation day 21).

Analysis of cortical differentiation

It was done using Immunofluorescence staining.

Appendix E: MATLAB code to count the number of cells

The following code was used to count the number of cells and to calculate the ratio of cells that showed positive cells compared to the total number of cells present. Otsu's algorithm (in-built MATLAB function) was used to calculate the threshold.

```
clc;
clear all;
close all;
% Reading image
gfapPLL5=imread('well 21 gfap.jpg');
figure(1);
imshow(gfapPLL5);
% Finding threshold of image to filter and convert image to binary
level=graythresh(gfapPLL5);
test=im2bw(gfapPLL5,level);
% Filtering noise from image
test1=bwmorph(test,'clean');
% Counting cells
[L num1]=bwlabel(~test1);
disp(num1);

% Reading image for dapi
dapiwell13=imread('well 21 dapi.jpg');
figure(3);
imshow(dapiwell13);
% Finding threshold of image to filter and convert image to binary
level=graythresh(dapiwell13);
test=im2bw(dapiwell13,level/7);
```

```
% Filtering noise from image
test1=bwmorph(test,'erode');
% Counting cells
[L num2]=bwlabel(~test1);
disp(num2);


disp('ratio is ')
disp(num1/num2)
```